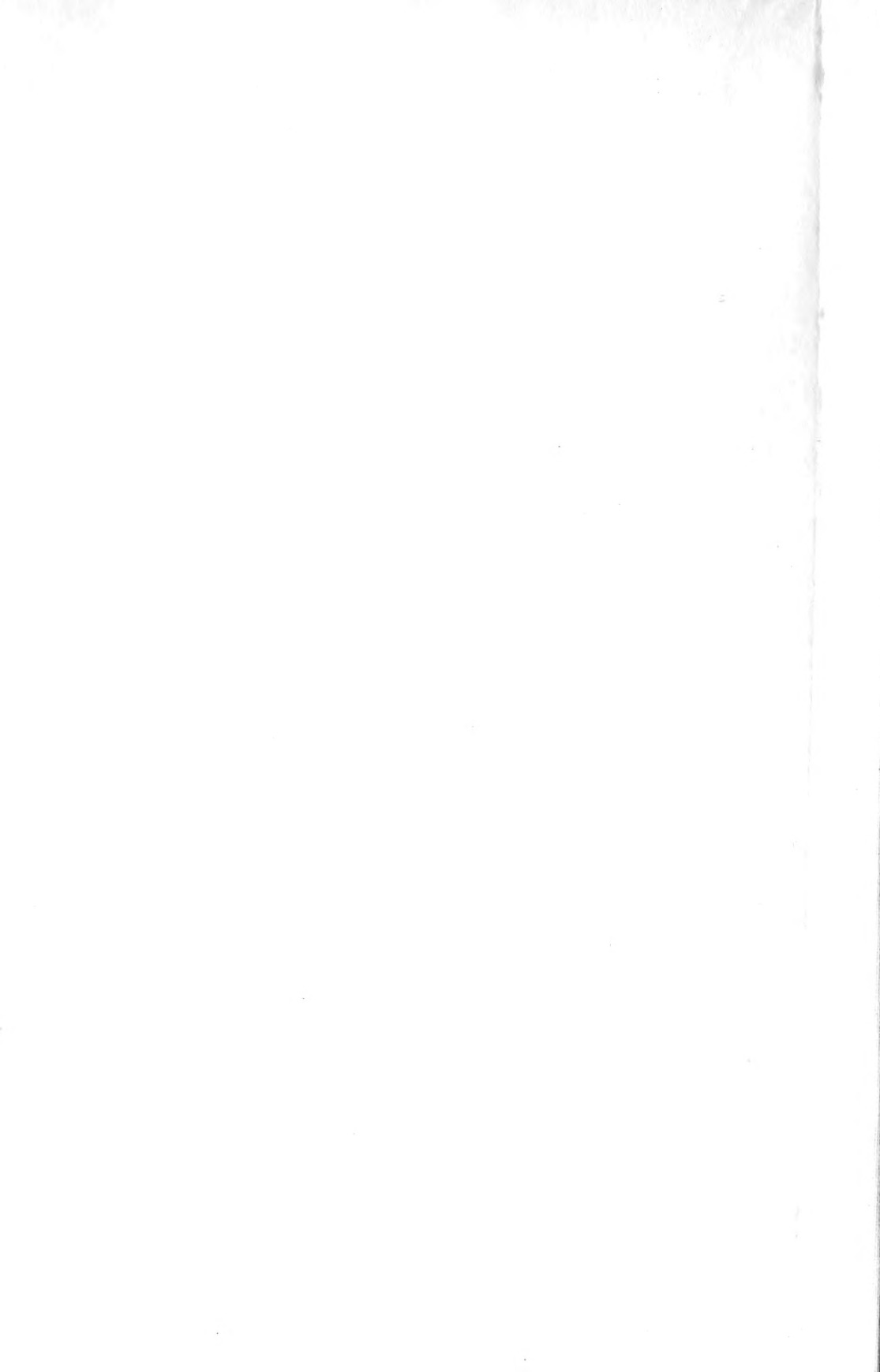




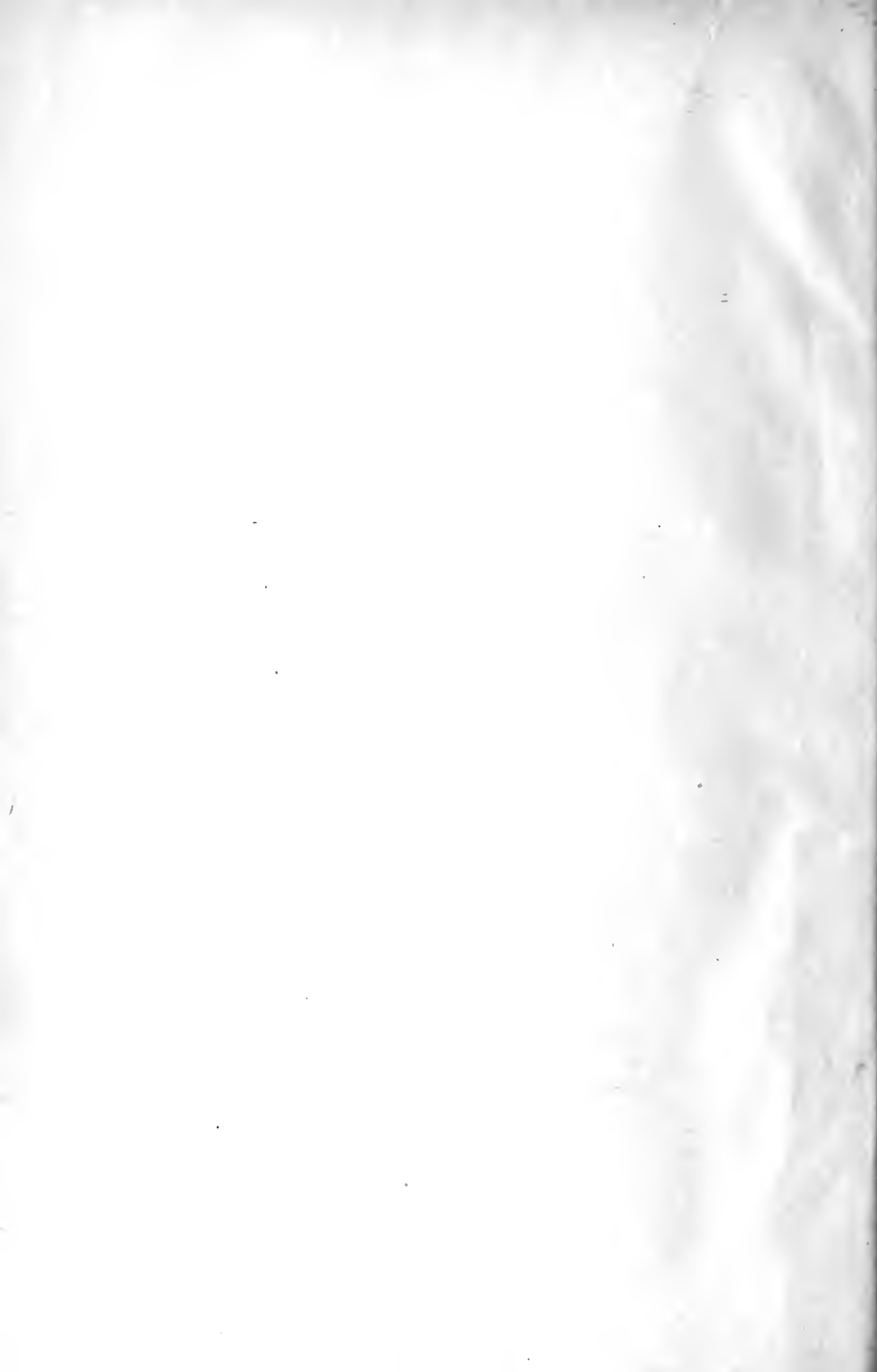




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THE JOURNAL OF  
EXPERIMENTAL MEDICINE.



# THE JOURNAL OF EXPERIMENTAL MEDICINE

EDITED BY  
SIMON FLEXNER, M.D.

VOLUME TWENTY-FIRST  
WITH SIXTY-SEVEN PLATES AND TWENTY-SIX  
FIGURES IN THE TEXT



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# CONTENTS.

NO. 1, JANUARY 1, 1915.

	PAGE
OPIE, EUGENE L., and ALFORD, LELAND B. The Influence of Diet upon Necrosis Caused by Hepatic and Renal Poisons. Part I. Diet and the Hepatic Lesions of Chloroform, Phosphorus, or Alcohol .....	1
OPIE, EUGENE L., and ALFORD, LELAND B. The Influence of Diet upon Necrosis Caused by Hepatic and Renal Poisons. Part II. Diet and the Nephritis Caused by Potassium Chromate, Uranium Nitrate, or Chloroform .....	21
PETROFF, S. A. A New and Rapid Method for the Isolation and Cultivation of Tubercle Bacilli Directly from the Sputum and Feces .....	38
AUER, JOHN. The Functional Effect of Experimental Intraspinal Injections of Sera with and without Preservatives . Plates I to II.	43
LEWIS, PAUL A., and MARGOT, ARTHUR GEORGES. The Function of the Spleen in the Experimental Infection of Albino Mice with <i>Bacillus tuberculosis</i> . Second Paper .....	84
FLEXNER, SIMON, NOGUCHI, HIDEYO, and AMOSS, HAROLD L. Concerning Survival and Virulence of the Microörganism Cultivated from Poliomyelitic Tissues. .... Plates 12 to 17.	91

NO. 2, FEBRUARY 1, 1915.

SMYTH, HENRY FIELD. The Reactions between Bacteria and Animal Tissues under Conditions of Artificial Cultivation Plates 18 to 20.	103
DOCHEZ, A. R., and AVERY, O. T. Varieties of Pneumococcus and Their Relation to Lobar Pneumonia .....	114
AVERY, OSWALD T. The Distribution of the Immune Bodies Occurring in Antipneumococcus Serum .....	133

LYALL, HAROLD W. The Types of Pneumococci in Tuberculous Sputum .....	146
FLEISHER, MOYER S., and LOEB, LEO. Further Investigations on the Mode of Action of Substances Inhibiting Tumor Growth and on Immunization against these Substances ..	155
HESELBERG, CORA. A Comparison of Autoplastic and Homeoplastic Transplantation of Thyroid Tissue in the Guinea Pig	164
CORPER, HARRY J. Notes on the Subcutaneous Absorption and the Quantitative Estimation of Cholesterol .....	179
GRAHAM, EVARTS A. The Resistance of Pups to Late Chloroform Poisoning in Its Relation to Liver Glycogen .....	185

### NO. 3, MARCH 1, 1915.

SPAIN, KATE C. The Relation between the Structure of the Epidermis of the Rat and the Guinea Pig, and the Proliferative Power of Normal and Regenerating Epithelial Cells of the Same Species .....	193
WHIPPLE, G. H., and SPEED, J. S. Liver Function as Influenced by Anesthetics and Narcotics .....	203
ZINSSER, HANS, HOPKINS, J. G., and GILBERT, RUTH. Notes on the Cultivation of <i>Treponema pallidum</i> .....	213
Plate 21. <i>revised 1915</i>	
BRONFENBRENNER, J. The Mechanism of the Abderhalden Reaction. Studies on Immunity. I .....	221
JOBLING, JAMES W., EGGSTEIN, A. A., and PETERSEN, WILLIAM. Serum Proteases and the Mechanism of the Abderhalden Reaction. Studies on Ferment Action. XX ....	239
TERRY, B. T. The Influence That Serum Exerts upon Trypanosomes, with Special Reference to Its Use for Experiments <i>in Vitro</i> with Atoxyl and Paraminophenylarsenoxyl	250
TERRY, B. T. Different Amounts of Transformed Atoxyl Produced by Incubating One Per Cent. and Ten Per Cent. Atoxyl in Blood .....	258
TERRY, B. T. The Effect of Heat on the Transforming and Binding Power of Blood .....	267
ROBERTSON, T. BRAILSFORD, and BURNETT, THEODORE C. The Influence of the Anterior Lobe of the Pituitary Body upon the Growth of Carcinomata .....	280



No. 4, APRIL 1, 1915.

PEARCE, LOUISE. A Comparison of Adult and Infant Types of Gonococci .....	289
KLINE, B. S., and WINTERNITZ, M. C. Studies upon Experimental Pneumonia in Rabbits. VII. The Production of Lobar Pneumonia .....	304
KLINE, B. S., and WINTERNITZ, M. C. Studies upon Experimental Pneumonia in Rabbits. VIII. <i>Intra Vitam</i> Staining in Experimental Pneumonia, and the Circulation in the Pneumonic Lung .....	311
WINTERNITZ, M. C., and KLINE, B. S. Studies upon Experimental Pneumonia in Rabbits. IX. The Part of the Leucocyte in the Immunity Reaction .....	320
BARBOUR, HENRY G., and PRINCE, ALEXANDER L. The Influence of Epinephrin upon the Coronary Circulation of the Monkey .....	330
Plate 22. <i>figs. 1-5</i>	
HESS, ALFRED F. A Test for Antithrombin in the Blood ..	338
BROWN, WADE H. Concerning Changes in the Biological Properties of <i>Trypanosoma lewisi</i> Produced by Experimental Means, with Especial Reference to Virulence ....	345
RETTGER, LEO F. The Influence of Milk Feeding on Mortality and Growth, and on the Character of the Intestinal Flora	365
GAY, FREDERICK P., and CHICKERING, HENRY T. Concentration of the Protective Bodies in Antipneumococcus Serum by Means of Specific Precipitation .....	389

No. 5, MAY 1, 1915.

HURWITZ, S. H., and DRINKER, C. K. The Factors of Coagulation in the Experimental Aplastic Anemia of Benzol Poisoning, with Special Reference to the Origin of Prothrombin .....	401
OLIVER, JEAN. The Histogenesis of Chronic Uranium Nephritis with Especial Reference to Epithelial Regeneration	425
Plates 23 to 30. <i>figs. 1-10</i>	
MARINE, DAVID. Observations on the Etiology of Goitre in Brook Trout. IV. The Effect of Feeding with Fresh and Stale Liver .....	452

BAITSELL, GEORGE A. The Origin and Structure of a Fibrous Tissue Which Appears in Living Cultures of Adult Frog Tissues .....	455
Plates 31 to 36.	
BRONFENBRENNER, J. The Nature of Anaphylatoxin. Studies on Immunity. II .....	480
HARRIS, WILLIAM H., and WADE, H. WINDSOR. The Wide-Spread Distribution of Diphtheroids and Their Occurrence in Various Lesions of Human Tissues .....	493
Plate 37.	
FLEXNER, SIMON, and AMOSS, HAROLD L. Diffusion and Survival of the Poliomyelitic Virus .....	509
FLEXNER, SIMON, and AMOSS, HAROLD L. The Rapid Production of Antidysenteric Serum .....	515
No. 6, JUNE 1, 1915.	
INGIER, ALEXANDRA. A Study of Barlow's Disease Experimentally Produced in Fetal and New-Born Guinea Pigs ..	525
Plate 38.	
NOGUCHI, HIDEYO. Pure Cultivation <i>in Vivo</i> of Vaccine Virus Free from Bacteria .....	539
Plates 39 to 50.	
FUNK, CASIMIR. The Transplantation of Tumors to Foreign Species. ....	571
Plate 51.	
FUNK, CASIMIR. The Effect of Arsenic Compounds on the Rous Chicken Sarcoma .....	574
ZINSSER, HANS, and HOPKINS, JOSEPH GARDNER. Antibody Formation against <i>Treponema pallidum</i> —Agglutination .	576
Plates 52 to 54.	
LAWSON, MARY R. Adult Tertian Malarial Parasites Attached to Peripheral Corpuscular Mounds. The Extracellular Relation of the Parasites to the Red Corpuscles.....	584
Plates 55 to 58.	
COHN, ALFRED E., FRASER, FRANCIS R., and JAMIESON, ROSS A. The Influence of Digitalis on the T Wave of the Human Electrocardiogram .....	593
Plates 59 to 64.	

KIRKBRIDE, MARY BUTLER. A Study of the Effect of Sensitization on the Development of the Lesions of Experimental Pneumonia in the Rabbit .....	605
FUNKHOUSER, EDGAR BRIGHT. The Visual Cortex, Its Localization, Histological Structure, and Physiological Function	617
LARSON, W. P., and BELL, E. T. A Study of the Pathogenic Properties of <i>Bacillus proteus</i> . ....	629
Plates 65 to 67.	



# THE INFLUENCE OF DIET UPON NECROSIS CAUSED BY HEPATIC AND RENAL POISONS.

## PART I. DIET AND THE HEPATIC LESIONS OF CHLOROFORM, PHOSPHORUS, OR ALCOHOL.\*

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(From the Pathological Laboratory of Washington University Medical School, St. Louis.)

Wide variation in the susceptibility of different individuals to the same poison has suggested the possibility that an apparently unrelated factor, such as diet, may modify the effect of a toxic substance. Individual susceptibility is illustrated by the occurrence of delayed chloroform poisoning. It is well known that chloroform administered to animals causes necrosis of the central portion of each hepatic lobule, and the severity of the lesion is roughly proportional to the quantity of chloroform which has been administered. Nevertheless, experiments in which chloroform has been administered to various animals during the course of other investigations have shown wide variation in the susceptibility of different individuals of the same species. Quantities which when given by mouth usually have little effect, occasionally produce death with multiple hemorrhage or jaundice, and at autopsy the liver shows widespread necrosis. The experiments which will be described furnish no comprehensive explanation of such individual susceptibility, but suggest that diet may have a profound influence upon the severity of intoxication and of disease.<sup>1</sup>

Foster<sup>2</sup> has described experiments to determine if the toxicity of ricin may be modified by diet. Three dogs kept upon a diet rich in protein died after the administration of 1 mg. of ricin per kilo, whereas two of three dogs upon a

\* Received for publication, October 6, 1914.

<sup>1</sup> Some of these experiments have been described (Opie, E. L., and Alford, L. B., *Jour. Am. Med. Assn.*, 1914, lxii, 895; 1914, lxiii, 136).

<sup>2</sup> Foster, N. B., *Proc. Soc. Exper. Biol. and Med.*, 1909, vi, 76; *Jour. Biol. Chem.*, 1909-10, vii, 379.

diet poorer in protein survived this dose. Foster does not believe that the diet caused the greater mortality of dogs which received protein in abundance, because these animals were given relatively larger doses of ricin.

An investigation of the effect of diet upon resistance to intoxication has been made by Hunt<sup>3</sup> who studied the behavior of acetonitril in mice and guinea pigs fed upon various substances. Certain diets, notably dextrose, oatmeal, liver, and kidney, greatly increase the resistance of mice to acetonitril. Hunt has previously found that thyroid fed to mice increases their resistance to acetonitril and suggests that increased resistance produced by diet may be due to the effect of diet upon the thyroid gland. Certain diets, notably eggs, milk, cheese, and various fats, greatly lower the resistance of mice to acetonitril. He refers increased susceptibility to increased disintegration of the acetonitril molecule with formation of hydrocyanic acid. Rats upon a diet of bread were slightly more susceptible to morphin than those fed upon oats.

The purpose of the present studies has been to determine if diet can influence the incidence or extent of a readily demonstrable lesion. Lesions of the liver have been produced by substances which have a peculiar affinity for the parenchymatous cells of the organ. Chloroform administered by inhalation, by mouth, or by subcutaneous injection causes necrosis of the hepatic cells in the center of each lobule of the liver and may implicate four fifths of each hepatic lobule. Fatty degeneration may occur in the heart muscle, in the kidney, or in other organs, and necrosis of cells of the convoluted tubules of the kidney is observed (rats), but the substance exhibits a selective action destroying the hepatic cells and leaving other tissues by comparison little altered. Phosphorus has been selected as another example of substances which exhibit an affinity for the liver; it produces fatty degeneration which is more advanced in the liver than in other organs. Human pathology suggests that alcohol under certain conditions may have a similar selective action, but experimental studies do not furnish convincing evidence that alcohol causes a distinctive lesion of the liver.

The experiments which will be described have dealt with substances which act as hepatic poisons. In a second group of experiments substances which exhibit a selective destructive action upon the kidney have been used. Both potassium chromate and uranium nitrate cause necrosis of the cells of the renal tubules but produce no necrosis of the liver or of other organs. It is well

<sup>3</sup> Hunt, R., *Bull. Hyg. Lab., U. S. P. H. and M.-H. S.*, 1911, No. 69.

known that these substances which have been widely used for the experimental production of nephritis cause albuminuria and the formation of urinary casts. They have been selected as examples of renal poisons.

#### METHOD.

White rats were used because they are omnivorous and eat readily various foods offered to them. They may be obtained in large number and are readily kept in good health, so that a considerable number of animals may be used for each test. They may be maintained in good condition and breed actively upon a diet consisting almost wholly of cereal, such as oats; animals upon a diet of oats eat meat eagerly. Rabbits are forced to eat protein with much difficulty, whereas carnivorous animals resist the administration of a carbohydrate diet. The rat, of which the diet exhibits a closer resemblance to that of man, is better able to adapt itself to variations of diet than either herbivorous or carnivorous animals.

In many of the experiments rats which as stock animals had previously received a diet consisting in large part of oats and bread were given during a short period either (a) carbohydrate in large part in the form of rolled oats and cane sugar in lumps, the latter being eaten in considerable quantities, (b) meat in the form of pig's heart or beefsteak, or (c) fat. The fatty diet consisted of beef fat alone fed during a period of several days before injection of the substance of which the toxicity was tested, or beef fat intimately mixed with rolled oats, or in some instances cottonseed oil administered by mouth to animals which were receiving the ordinary diet of oats. At the end of a period varying from three to seven days the toxic substance was administered by subcutaneous injection. In some instances the special diet was continued after administration of the toxic substance. In other instances the special diet was discontinued, and since the period of special diet was short there was little opportunity for the harmful effect of those deficiencies in diet which are demonstrable after periods not less than from four to six weeks, in birds by neuritis and in small mammals by scurvy. The weight of each animal was recorded at the time when special diet was begun and again several days later when the toxic substance was administered. The body-weight serves as an index of the effect of the diet upon the nutrition of the animal.

In the following experiments dosage has been adjusted to body-weight and in each series of experiments the smallest dose has been given to the smallest animal, the dose increasing in the series with the weight of the animals.

The number of animals which have died in any group of specially dieted animals is an index of variations in toxicity referable to diet. The average duration of life of animals which have died serves as a further indication of variations in toxicity, subordinate in importance to the number of fatalities. Pathological changes in the internal organs have proved an additional index to the influence of diet upon the severity of intoxication.

## EFFECT OF DIET UPON THE LIVER.

Since the liver serves as a storehouse for fat and for carbohydrates in the form of glycogen, preliminary experiments have been made to determine the effect of various diets which have been employed upon the liver of the white rat. Chalataw<sup>4</sup> found that sunflower-seed oil, cod liver oil, or beef fat fed to white rats produced no injurious effect. Moderate fatty infiltration of the liver was found after seven days; it persisted during two months and subsequently diminished, disappearing at the end of about four months. We have examined the livers of white rats which have received beef fat during eight days. Fat is present throughout the tissue, but is most abundant about the central part of each lobule, where, surrounding the central vein in an area occupying approximately one half the radius from the central vein to the portal space, the cells are filled with droplets staining with Sudan III. A similar condition was found after fat diet received during five days. The livers of animals fed upon meat or oats and sugar exhibit no accumulation of visible fat. In the liver of animals fed with oats and cane sugar during five and eight days, Best's carmine stain demonstrated the presence of glycogen in large quantity; abundant throughout the lobule it is present, unlike fat, in greatest quantity in those cells which are in contact with the portal spaces.

## CHLOROFORM.

In the following experiments chloroform was administered to animals which had been subjected to the diets previously enumerated. Chloroform was mixed with two parts of sterile paraffin oil, selected because it is absorbed with difficulty. A preliminary experiment showed that a dose approximately 0.2 of a cubic centimeter per 100 grams of body-weight injected subcutaneously failed to kill, whereas from 0.3 to 0.5 of a cubic centimeter per 100 grams killed after four days, 0.6 of a cubic centimeter after two days, and 0.7 of a cubic centimeter within one day. Irregularities in the action of the poison are occasionally seen.

In the series of experiments recorded in table I animals were given during four days (1) oats and cane sugar, (2) meat (pig's heart), or (3) fat (beef fat).

<sup>4</sup> Chalataw, S. S., *Virchows Arch. f. path. Anat.*, 1912, ccvii, 453.



TABLE I.  
*Chloroform.*

Diet.	Weight before diet.	Weight after 4 dys.	Dose per 100 gm.	Length of life.	Remarks.
Oats and sugar	140 gm.	145 gm.	0.1 c.c.	*	
	145 gm.	142 gm.	0.15 c.c.	3 dys.	Liver: necrosis $\frac{1}{3}$ lobule.
	147 gm.	153 gm.	0.2 c.c.	7 dys.	No necrosis of liver; nephritis.
	155 gm.	147 gm.	0.25 c.c.	*	
	180 gm.	177 gm.	0.3 c.c.	4 dys.	Necrosis $\frac{1}{4}$ lobule.
Average duration of survival after carbohydrate diet..... $4\frac{2}{3}$ dys.					
Meat	140 gm.	142 gm.	0.1 c.c.	3 dys.	Necrosis $\frac{3}{4}$ lobule.
	152 gm.	165 gm.	0.15 c.c.	4 dys.	Necrosis $\frac{1}{4}$ lobule.
	162 gm.	160 gm.	0.2 c.c.	3 dys.	Necrosis $\frac{1}{2}$ lobule.
	165 gm.	165 gm.	0.25 c.c.	2 dys.	Necrosis $\frac{1}{2}$ lobule.
	140 gm.	142 gm.	0.3 c.c.	3 dys.	Necrosis $\frac{1}{2}$ lobule.
Average duration of survival after meat diet..... 3 dys.					
Fat	140 gm.	150 gm.	0.1 c.c.	2 dys.	Necrosis $\frac{3}{4}$ lobule.
	155 gm.	150 gm.	0.15 c.c.	1 dy.	Necrosis $\frac{3}{4}$ lobule.
	160 gm.	157 gm.	0.2 c.c.	2 dys.	Necrosis $\frac{3}{4}$ lobule.
	172 gm.	175 gm.	0.25 c.c.	2 dys.	Necrosis $\frac{1}{2}$ lobule.
	180 gm.	175 gm.	0.3 c.c.	2 dys.	Necrosis $\frac{1}{2}$ lobule.
Average duration of survival after fat diet..... $1\frac{4}{5}$ dys.					

\* = lived.

Two of five animals which received carbohydrates survived, whereas all of those which received meat or fat died. Differences in toxicity after different diets is further shown by the average duration of life of animals which died. All animals which had received fat died within one or two days, whereas those which received meat lived from two to four days.

Microscopic examination of the livers of animals which have died furnishes evidence that these differences in susceptibility induced by diet are associated with corresponding differences in the severity of the characteristic lesion of chloroform poisoning, namely, necrosis of the liver. Three animals which received carbohydrate diet died, but only two exhibited necrosis of the liver. In these two animals necrosis occurred immediately about the central vein and implicated an area extending from one fourth to one third the dis-

tance from central vein to portal space (designated roughly in the table as one fourth or one third of the liver lobule). The necrotic parenchymatous cells have undergone disintegration and have been in large part removed and replaced by loose tissue composed of capillaries and mononuclear cells. With the more severe necrosis which has occurred in animals fed with meat or fat a greater part of the lobule is implicated, the hepatic cells have undergone coagulative necrosis and remain as columns of hyaline material between the capillaries; absorption of necrotic cells has not begun.

The animal which received carbohydrate diet and died seven days after administration of chloroform exhibited nephritis. The kidneys were large and bright yellow. The cells of the convoluted tubules are vacuolated, the interstitial tissue in places contains mononuclear cells, and casts are numerous within the tubules.

TABLE II.

*Chloroform.*

Diet.	Weight before diet.	Weight after 6 dys.	Dose per 100 gm.	Length of life.	Remarks.
Oats and sugar	110 gm.	102 gm.	0.05 c.c.	*	Slight necrosis of liver; nephritis.
	122 gm.	120 gm.	0.1 c.c.	10 dys.	
	135 gm.	130 gm.	0.15 c.c.	*	
	140 gm.	142 gm.	0.2 c.c.	*	
	165 gm.	160 gm.	0.25 c.c.	*	
Average duration of survival with carbohydrate diet.....10+dys.					
Meat	110 gm.	110 gm.	0.05 c.c.	11 dys.	No necrosis of liver; nephritis.
	130 gm.	130 gm.	0.1 c.c.	*	
	137 gm.	137 gm.	0.15 c.c.	*	Necrosis of $\frac{3}{4}$ lobule. Necrosis of $\frac{3}{4}$ lobule.
	145 gm.	144 gm.	0.2 c.c.	3 dys.	
	160 gm.	168 gm.	0.25 c.c.	3 dys.	
Average duration of survival with meat diet..... $5\frac{2}{3}$ +dys.					
Fat	132 gm.	115 gm.	0.025 c.c.	*	Necrosis of $\frac{3}{4}$ lobule. Necrosis of $\frac{1}{2}$ lobule. Necrosis of $\frac{1}{2}$ lobule. Necrosis of $\frac{1}{2}$ lobule. Necrosis of $\frac{1}{2}$ lobule.
	115 gm.	115 gm.	0.05 c.c.	3 dys.	
	135 gm.	125 gm.	0.1 c.c.	1 dy.	
	157 gm.	145 gm.	0.15 c.c.	1 dy.	
	167 gm.	148 gm.	0.2 c.c.	2 dys.	
	215 gm.	200 gm.	0.25 c.c.	2 dys.	
Average duration of survival with fat diet..... $1\frac{1}{3}$ dys.					

\* = lived.

Since all of the animals which received meat or fat died, it was assumed that smaller doses of chloroform might demonstrate greater differences in toxicity referable to diet. In the preceding series (table II) the special diets previously enumerated were given during six days. The smallest dose of chloroform given to animals which had received fat was 0.025 of a cubic centimeter, but in view of the foregoing experiment, the smallest doses to animals which had received carbohydrates or meat were placed at 0.05 of a cubic centimeter.

The mortality and the average duration of life of animals receiving carbohydrates, meat, or fat confirm the results obtained in the first series of experiments. Susceptibility to intoxication is greatest after a diet of fat, less after meat, and least in animals which have received oats and cane sugar. Two animals died after ten or eleven days; one showed disintegration and absorption of a few hepatic cells immediately about the central vein of each lobule, the other exhibited no necrosis of the liver. In both animals there was advanced nephritis with large yellow kidneys containing casts. It may be assumed that these animals did not die as the result of necrosis of the liver but in consequence of nephritis which (as in one animal of table I) progresses more slowly than the hepatic lesion. If these two animals are excluded, the influence of various diets upon the occurrence of hepatic necrosis is clearly definable, for fatal necrosis of the liver after a diet of fat has been produced by a dose of chloroform (0.05 of a cubic centimeter), one fourth of that (0.2 of a cubic centimeter) which produces the same lesion after a diet of meat, and more than one fifth of that (0.25 of a cubic centimeter +) needed to produce the lesion after a diet of oats and sugar.

In the first series of experiments (table I) animals which received oats and sugar during four days lost no weight, those which received meat gained three grams, and those which received fat maintained their weight. In the second series of experiments (table II) animals which received oats and sugar during six days lost an average of four grams each, those on meat maintained their weight, and those on fat lost an average of fifteen grams each. Since this loss of weight indicates that fat continued during a pro-

longed period is an insufficient diet, the experiment was repeated by adding fat to a carbohydrate diet. Animals received (a) oats and sugar, (b) meat alone. One group of animals received (c) beef fat inseparably ground with rolled oats; another group was fed upon (d) rolled oats and received daily one cubic centimeter of cottonseed oil administered by mouth (table III).

TABLE III.

*Chloroform.*

Diet.	Weight before diet.	Weight after 5 dys.	Dose per 100 gm.	Length of life.	Remarks.
Oats and sugar	78 gm.	78 gm.	0.05 c.c.	*	
	91 gm.	101 gm.	0.1 c.c.	*	
	97 gm.	102 gm.	0.15 c.c.	*	
	95 gm.	104 gm.	0.2 c.c.	*	
	108 gm.	115 gm.	0.25 c.c.	*	
Meat	83 gm.	83 gm.	0.05 c.c.	2 dys.	Necrosis of $\frac{2}{3}$ lobule of liver.
	80 gm.	80 gm.	0.1 c.c.	5 dys.	Necrosis of $\frac{1}{3}$ lobule.
	87 gm.	105 gm.	0.15 c.c.	3 dys.	Necrosis of $\frac{1}{3}$ lobule.
	95 gm.	101 gm.	0.2 c.c.	3 dys.	Necrosis of $\frac{1}{3}$ lobule.
	110 gm.	110 gm.	0.25 c.c.	*	
Average..... $4\frac{1}{4}$ +dys.					
Oats and fat	91 gm.	85 gm.	0.05 c.c.	2 dys.	Liver not examined.
	90 gm.	96 gm.	0.1 c.c.	*	
	88 gm.	92 gm.	0.15 c.c.	2 dys.	Necrosis of $\frac{1}{2}$ lobule.
	95 gm.	98 gm.	0.2 c.c.	2 dys.	Necrosis of $\frac{1}{2}$ lobule.
	110 gm.	114 gm.	0.25 c.c.	2 dys.	Necrosis of $\frac{1}{2}$ lobule.
Average..... 2 +dys.					
Oats and cottonseed oil	73 gm.	77 gm.	0.05 c.c.	*	
	79 gm.	80 gm.	0.1 c.c.	2 dys.	Necrosis of $\frac{2}{3}$ lobule.
	85 gm.	81 gm.	0.15 c.c.	2 dys.	Necrosis of $\frac{1}{2}$ lobule.
	80 gm.	82 gm.	0.2 c.c.	2 dys.	Necrosis of $\frac{1}{2}$ lobule.
	102 gm.	100 gm.	0.25 c.c.	4 dys.	
Average..... $2\frac{1}{2}$ +dys.					

\* = lived.

The favorable influence of a carbohydrate diet is evident. The difference in the effect of meat and of fat is less conspicuous than in the former series, doubtless for the reason that fat has been combined with carbohydrate. In this series animals which received oats and sugar gained in weight an average of six grams each; those which received meat, five grams each; those which received oats and

fat, two grams each; those which received oats and cottonseed oil maintained their weight.

The experiments have shown that chloroform is more toxic to animals which have received meat or fat than to those which have received a diet composed in large part of carbohydrates. The following experiment (table IV) suggests that the toxicity of chloroform in animals which have received a mixture of meat and fat is perhaps even greater than the toxicity tested in animals which have received either meat or fat alone. Ten animals received during six days a mixture consisting of equal parts of beef fat and pig's heart. The average weight of these animals showed no material change (an average loss of two grams each).

TABLE IV.  
*Chloroform.*

Diet.	Chloroform per 100 gm. of body-weight.	Length of life after administration.
Fat and meat	0.05 c.c.	2 dys.
	0.05 c.c.	3 dys.
	0.075 c.c.	2 dys.
	0.075 c.c.	1 dy.
	0.1 c.c.	2 dys.
	0.1 c.c.	2 dys.
	0.125 c.c.	2 dys.
	0.125 c.c.	2 dys.
	0.15 c.c.	2 dys.
	0.15 c.c.	2 dys.
		Average... 2 dys.

Comparison with previous experiments shows that the corresponding doses of chloroform have not caused the same uniform mortality when administered to animals upon a diet of fat or of meat alone; approximately one fifth (0.05 of a cubic centimeter) of the lethal dose for animals upon a carbohydrate diet (0.25 to 0.3 of a cubic centimeter) has caused death.

When animals have been given small doses of chloroform (0.025 to 0.25 of a cubic centimeter), those which have received a carbohydrate diet have usually survived. With larger doses (table I) differences in the extent of necrosis after carbohydrate diet on the one hand, and meat or fat on the other hand, have already been noted. No noteworthy difference between the extent of necrosis

with meat and with fat has been found (tables I, II, and III). In the following experiment performed at the beginning of the present investigation an accurate record of the period of survival after administration of chloroform was not kept; histological examination of the liver in this series of experiments further demonstrates that the severity of necrosis is influenced by diet (table V).

TABLE V.

*Chloroform.*

Diet.	Weight.	Dose per 100 gm.	Length of life.	Remarks.
Oats and sugar	257 gm.	0.1 c.c.	*	
	230 gm.	0.2 c.c.	*	
	240 gm.	0.3 c.c.	Within 4 dys.	Necrosis $\frac{1}{5}$ liver lobule; complete absorption.
	245 gm.	0.4 c.c.	Within 4 dys.	Necrosis $\frac{1}{5}$ lobule; incomplete absorption.
	215 gm.	0.5 c.c.	Within 5 dys.	Necrosis $\frac{1}{5}$ lobule; complete absorption.
Oats and meat	247 gm.	0.1 c.c.	*	
	238 gm.	0.2 c.c.	Within 4 dys.	Necrosis $\frac{1}{5}$ liver lobule; no absorption.
	220 gm.	0.3 c.c.	Within 4 dys.	Necrosis $\frac{1}{5}$ liver lobule; no absorption.
	230 gm.	0.4 c.c.	Within 4 dys.	Necrosis $\frac{1}{5}$ liver lobule; no absorption.
	240 gm.	0.5 c.c.	Within 4 dys.	Necrosis $\frac{1}{5}$ liver lobule; no absorption.

\* = lived.

Difference in the character of the lesion noted in the two groups is even more marked than the extent of necrosis (necrosis of one fifth of liver lobules in the tables means necrosis in an area about the central vein one fifth of the distance from the central vein to the portal space). In all of the animals which received oats and sugar, necrotic cells surrounding each central vein have undergone disintegration and cell debris has disappeared wholly or in considerable part; whereas in the animals which have received a diet containing meat, hyaline cells devoid of nuclei have maintained their shape and form columns between capillaries of which the endothelium is wholly or partially preserved. With the more complete necrosis caused by chloroform after a diet of meat, necrosis is perhaps so profound that the inflammatory reaction which brings about absorption of dead cellular elements is paralyzed.

A conspicuous result of the experiments which have been described is the demonstration of the susceptibility to chloroform

induced by a diet containing fat in large amount. Fatal necrosis of the liver after a diet of fat occurs with doses which produce little effect upon animals fed with carbohydrates or meat. Control experiments have shown that the liver of white rats fed with fat contains visible fat stainable by Sudan III, deposited in greatest abundance about the central vein of each lobule. Necrosis caused by chloroform occurs in the same situation. In view of the readiness with which chloroform is dissolved in fat it is probable that increased fat favors the fixation of chloroform within the hepatic cells; chloroform so fixed exerts its injurious action upon the protoplasm of the cell.

In accordance with the well known theory of Meyer<sup>5</sup> and Overton<sup>6</sup> chloroform produces anesthesia because it is a fat solvent and is taken up by the lipoids of nervous tissue. Chloroform is distributed in the blood and in the tissues in accordance with its solubility in the substances with which it comes into contact. Wells<sup>7</sup> has offered the suggestion that a fatty liver might absorb more chloroform from the blood than a normal liver.

Observations of Lattes<sup>8</sup> are noteworthy; he found that finely emulsified fat injected into the veins of dogs diminished more than one half the quantity of chloroform needed to produce narcosis when simultaneously injected into the vein. When fat was injected a smaller quantity of chloroform was needed to cause disappearance of reflexes, and the period during which an animal could be kept under anesthesia by a given quantity of chloroform was prolonged. Similar diminution of the dose required to produce a given result occurred when fat was given by mouth. Lattes obtained evidence that the fat which he administered absorbed chloroform, and holding it in the blood serum diminished the quantity fixed at the beginning of narcosis by the adipose tissue of the body.

The fats used in the foregoing experiments have been beef fat, which consists of olein, stearin, and palmitin, and cottonseed oil, which is in large part olein. Experiments performed by several Russian observers have shown that cholesterolin and foods containing cholesterolin produce profound pathological changes in rabbits. Stuckey<sup>9</sup> noted the occurrence of arteriosclerosis in rabbits fed upon egg-yolk and brain tissue, although no lesions were found in animals which had received pure neutral fats. Chalataw<sup>10</sup> fed rabbits upon egg-yolk and brain tissue and noted the accumulation of doubly refractive fat droplets demonstrable in the liver by means of Nichol's prisms. Within thirty days after

<sup>5</sup> Meyer, H., *Arch. f. exper. Path. u. Pharmakol.*, 1899, xlii, 109.

<sup>6</sup> Overton, *Studien über die Narkose*, Jena, 1901.

<sup>7</sup> Wells, H. G., *Arch. Int. Med.*, 1908, i, 589.

<sup>8</sup> Lattes, L., *Gior. d. r. Accad. di med. di Torino*, 1910, xvi, 126; abstracted in *Jahresb. f. Thier-Chem.*, 1910, xl, 1199; *München. med. Wchenschr.*, 1910, lvii, 2084.

<sup>9</sup> Stuckey, N. W., *Centralbl. f. allg. Path. u. path. Anat.*, 1912, xxxiii, 910.

<sup>10</sup> Chalataw, S. S., *Virchows Arch. f. path. Anat.*, 1912, ccvii, 453.

the beginning of feeding, degenerative changes were found in the liver cells and there was increase of the interstitial tissue of the organ. Rabbits fed upon egg-white, cow's milk, meat juice, sunflower-seed oil, cod liver oil, and beef fat exhibited no injurious effects. Feeding of egg-yolk and brain substance to rats was followed by no accumulation of anisotropic fat in the liver and by no evidence of degenerative change in the hepatic cells. The effects of administration of the two anisotropic lipoids found in egg-yolk have been investigated. Wesselkin<sup>11</sup> fed pure lecithin to rabbits and obtained no evidence that it was stored in the liver. Anitschkow<sup>12</sup> maintained that pure cholesterol fed to rabbits causes arteriosclerosis. Chalатов<sup>13</sup> fed cholesterol to rabbits and obtained evidence that it accumulates in the liver, causing in some instances advanced cirrhosis.

TABLE VI.

*Chloroform.*

Diet.	Weight before diet.	Weight after 6 dys.	Dose per 100 gm.	Length of life.	Remarks.
Oats and sugar	82 gm.	90 gm.	0.025 c.c.	*	Scant necrosis of liver.
	88 gm.	95 gm.	0.075 c.c.	*	
	104 gm.	114 gm.	0.125 c.c.	4 dys.	
	122 gm.	116 gm.	0.175 c.c.	*	
	142 gm.	146 gm.	0.225 c.c.	*	
Average length of survival after carbohydrate diet. . . . . 4 + dys.					
Beef fat	76 gm.	87 gm.	0.025 c.c.	8 dys.	No necrosis of liver; nephritis.
	100 gm.	100 gm.	0.075 c.c.	3 dys.	Necrosis $\frac{2}{3}$ lobule.
	107 gm.	105 gm.	0.125 c.c.	6 dys.	Scant necrosis of liver; nephritis.
	102 gm.	108 gm.	0.175 c.c.	*	Necrosis $\frac{1}{4}$ lobule.
	155 gm.	162 gm.	0.225 c.c.	4 dys.	
Average length of survival after fatty diet. . . . . 5 $\frac{1}{4}$ + dys.					
Calf brain	77 gm.	78 gm.	0.025 c.c.	2 dys.	Necrosis $\frac{1}{3}$ lobule.
	98 gm.	94 gm.	0.075 c.c.	2 dys.	Necrosis $\frac{1}{2}$ lobule.
	102 gm.	100 gm.	0.125 c.c.	2 dys.	Necrosis $\frac{1}{2}$ lobule.
	111 gm.	108 gm.	0.175 c.c.	*	No necrosis of liver; nephritis.
	148 gm.	141 gm.	0.225 c.c.	4 dys.	
Average length of survival after diet of brain. . . . . 2 $\frac{1}{2}$ + dys.					

\* = lived.

Although the experiments just cited have demonstrated no pathological changes produced by cholesterol in omnivorous animals, such as the rat, it has seemed not improbable, in view of the in-

<sup>11</sup> Wesselkin, N. W., *Virchows Arch. f. path. Anat.*, 1913, ccxii, 225.

<sup>12</sup> Anitschkow, N. N., *Beitr. z. path. Anat. u. z. allg. Path.*, 1913, lvi, 379.

<sup>13</sup> Chalатов, S. S., *Beitr. z. path. Anat. u. z. allg. Path.*, 1913, lvii, 85.



creased toxicity to chloroform caused by a fatty diet, that food containing cholesterin might influence the toxicity of a substance which causes an hepatic lesion. White rats fed upon brain substance have been given subcutaneously doses of chloroform similar in amount to those previously employed. The animals eat this food eagerly and at least during the period of the experiment maintain their weight. In the following experiment the effect of diets consisting of (a) oats and sugar, (b) beef fat, and (c) brain of calf have been compared. The animals have received these diets during six days (table VI).

The toxicity of chloroform has been greater in animals fed upon brain than in those fed upon fat. The average length of life of animals which received fat was more than twice as great as that of animals which received brain. Animals which received carbohydrate gained in weight an average of 4.2 grams; those which received fat gained 4.4 grams; those which received brain lost an average of 3 grams.

For comparison with the foregoing series white rats were fed during seven days with boiled egg-yolk (table VII).

TABLE VII.

*Chloroform.*

Diet.	Weight before diet.	Weight after 7 dys.	Dose per 100 gm.	Length of life.	Remarks.
Egg-yolk	168 gm.	173 gm.	0.025 c.c.	2 dys.	Necrosis $\frac{2}{3}$ liver lobule.
	175 gm.	178 gm.	0.075 c.c.	3 dys.	Necrosis $\frac{2}{3}$ liver lobule.
	187 gm.	190 gm.	0.125 c.c.	3 dys.	Necrosis $\frac{1}{2}$ liver lobule.
	183 gm.	190 gm.	0.175 c.c.	3 dys.	Necrosis $\frac{1}{2}$ liver lobule.
	187 gm.	191 gm.	0.225 c.c.	3 dys.	Necrosis $\frac{1}{3}$ liver lobule.

Egg-yolk, which contains 35 per cent. of fat, including cholesterin and lecithin, induces toxicity equal to that found with brain substance. Animals which received egg-yolk during seven days gained in weight an average of 4.4 grams. These experiments with substances containing fat and cholesterin confirm the results obtained with beef fat and olive oil. Further experiments with pure cholesterin, given to animals receiving a sustaining diet, will be required to determine if the somewhat greater toxicity found after diets of brain and egg-yolk is referable to cholesterin.

## PHOSPHORUS.

The influence of diet upon the toxicity of phosphorus was next tested because the substance exerts a selective action on the liver, producing profound injury to the cells of the organ. Fatty degeneration produced by phosphorus is not limited to the cells of the liver,

TABLE VIII.

*Phosphorus.*

Diet.	Weight before diet.	Weight after 6 dys.	Dose per 100 gm.	Length of life.	Remarks.
Oats and sugar	115 gm.	111 gm.	0.05 c.c.	*	Scant fatty degeneration of liver; pregnant. Fatty degeneration; scant necrosis. Fatty degeneration; several foci of necrosis.
	128 gm.	117 gm.	0.06 c.c.	*	
	142 gm.	139 gm.	0.07 c.c.	2 dys.	
	168 gm.	158 gm.	0.08 c.c.	4 dys.	
	172 gm.	159 gm.	0.09 c.c.	4 dys.	
Average length of survival with carbohydrate diet. . . . . 3½ +dys.					
Meat	120 gm.	107 gm.	0.05 c.c.	11 dys.	Abscess of lungs; beginning proliferation of connective tissue of liver. Advanced fatty degeneration and necrosis of cells of liver; beginning proliferation of connective tissue. Fatty degeneration; focal necrosis of ½ tissue of liver. Fatty degeneration; focal necrosis of ¼ tissue of liver; proliferation of cells in connective tissue.
	130 gm.	112 gm.	0.06 c.c.	4 dys.	
	146 gm.	130 gm.	0.07 c.c.	4 dys.	
	152 gm.	137 gm.	0.08 c.c.	4 dys.	
	175 gm.	150 gm.	0.09 c.c.	2 dys.	
Average length of survival with meat diet. . . . . 5 dys.					
Fat	113 gm.	98 gm.	0.05 c.c.	*	Abscesses of lung; liver normal. Slight fatty degeneration.
	144 gm.	120 gm.	0.06 c.c.	*	
	135 gm.	125 gm.	0.07 c.c.	*	
	147 gm.	135 gm.	0.08 c.c.	4 dys.	
	193 gm.	172 gm.	0.09 c.c.	2 dys.	
Average length of survival with fat diet. . . . . 3 +dys.					

\* = lived.

for the parenchymatous cells of the kidney, heart, muscle, and other organs show a similar change. A 1 per cent. solution of phosphorus in oil (oleum phosphoratum) has been injected subcutaneously. The oil injected with the phosphorus is negligible for the purpose

of the experiments since the lethal dose of oil of phosphorus for white rats has proved to be from 0.07 to 0.08 of a cubic centimeter per 100 grams of body-weight. Animals have been given during six days (a) oats and cane sugar, (b) pig's heart, or (c) beef fat; at the end of this time phosphorus has been injected. The special diet has been continued after administration of the substance (table VIII).

The weight of all animals in this series is apparently much diminished after receiving special diets, the average loss for animals on oats and sugar being 8.2 grams, on meat 17.4, and on fat 16.4. It is probable that this discrepancy occurs, in large part at least, because the second weight has been taken in the morning before the animals had been fed. It is noteworthy that the susceptibility to phosphorus bears no relation to diminution in weight. All animals which have received meat have died, but it is not improbable that the death of the animal which received the smallest dose of phosphorus is referable to suppurative bronchopneumonia. Death of one animal which received oats and sugar is perhaps in part referable to advanced pregnancy; of one animal which received fat, to bronchopneumonia with abscesses of the lungs.

In the following series of experiments beefsteak has been used in place of pig's heart given in the last series. A special diet has been given during six days and at the end of this time all animals have been allowed to eat oats freely (table IX).

The result of this series of experiments is identical with that of the foregoing series. All of the animals which received meat have died. Susceptibility to the poison bears no relation to the effect of diet upon body-weight; animals which received carbohydrate maintained their weight, gaining an average of 1.4 grams; animals with meat have, in great part, maintained their weight, losing an average of two grams, the loss being wholly due to the loss of weight of one animal; whereas animals on a diet of fat have lost an average of nine grams.

In both series of experiments a study of the pathological changes in the livers of animals which have died gives noteworthy confirmation of the increased susceptibility to phosphorus induced by a diet

TABLE IX.

*Phosphorus.*

Diet.	Weight before diet.	Weight after 6 dys.	Dose per 100 gm.	Length of life.	Remarks.
Oats and sugar	75 gm.	70 gm.	0.05 c.c.	*	Fatty degeneration of liver; no necrosis.
	85 gm.	85 gm.	0.06 c.c.	2 dys.	
	80 gm.	90 gm.	0.07 c.c.	*	Fatty degeneration; no necrosis.
	90 gm.	90 gm.	0.08 c.c.	2 dys.	
	98 gm.	100 gm.	0.09 c.c.	3 dys.	Fatty degeneration; no necrosis.
Average length of survival with carbohydrate diet . . . . . $2\frac{1}{3}$ + dys.					
Meat	65 gm.	60 gm.	0.05 c.c.	4 dys.	Fatty degeneration of liver; focal necrosis.
	70 gm.	74 gm.	0.06 c.c.	3 dys.	Fatty degeneration; focal necrosis; beginning proliferation of connective tissue.
	82 gm.	79 gm.	0.07 c.c.	3 dys.	Fatty degeneration; slight focal necrosis.
	85 gm.	87 gm.	0.08 c.c.	3 dys.	Fatty degeneration; focal necrosis of $\frac{1}{3}$ tissue.
	115 gm.	101 gm.	0.09 c.c.	1 dy.	
Average length of survival with meat diet . . . . . $2\frac{4}{5}$ dys.					
Fat	72 gm.	65 gm.	0.05 c.c.	*	Fatty degeneration of liver; no necrosis.
	77 gm.	65 gm.	0.06 c.c.	*	
	75 gm.	65 gm.	0.07 c.c.	3 dys.	
	82 gm.	73 gm.	0.08 c.c.	1 dy.	Fatty degeneration; no necrosis.
	132 gm.	125 gm.	0.09 c.c.	2 dys.	No change found in liver.
Average length of survival with fat diet . . . . . 2 + dys.					

\* = lived.

of meat. In white rats phosphorus in the doses which have been employed produces fatty degeneration, which makes its appearance at the end of one or two days, and after three or four days in some instances has reached such severity that every hepatic cell is distended with fat droplets. During the first two days or even later, when the change is slight, visible fat may be found in greatest abundance in the center of each lobule; but later, when the change is more advanced, fat is usually more abundant in the peripheral part of the lobules adjacent to the portal spaces, though occasionally fat, when in very large quantity, is equally distributed in all parts of the lobule

or is scattered in irregularly disposed patches. The effect of phosphorus upon the periphery of the lobule is more conspicuous when necrosis occurs. Coagulative necrosis of the hepatic cells has been observed in only two animals which have received a carbohydrate diet; these animals have survived four days and have received doses of phosphorus equal to 0.08 of a cubic centimeter of oleum phosphoratum. In one of two instances in which necrosis occurred with a carbohydrate diet several small foci were found below the capsule of the liver; in the other instance small areas of necrosis have occurred in contact with many of the portal spaces. In these areas liver cells have lost their nuclei, the cell protoplasm is hyaline, there is hemorrhage, and polynuclear leucocytes are fairly abundant. Cells with round and oval nuclei are numerous in the portal spaces, but there is no obvious proliferation of connective tissue. Necrosis was not found in the liver of any animal which had received a diet of fat.

In animals which have received meat, changes in the liver are far more profound. Rats which have lived three or four days show more intense fatty degeneration than those animals on other diets which have lived during a corresponding period, and necrosis, present in seven instances, has occurred in animals which have received from 0.05 to 0.08 of a cubic centimeter of oleum phosphoratum; in three instances necrosis has implicated from one to two fifths of the sectioned liver tissue. The tendency to localization in the periphery of the lobule in contact with the portal space is evident in every liver examined save one, but the even distribution which characterizes the relation of chloroform necrosis to the center of the lobule is absent, and foci of necrosis varying in size and shape may occur in any part of the lobule. In one instance necrotic tissue surrounded the central veins and irregular projections extended as far as the portal spaces.

Accumulation of cells occurs in the tissue of the portal spaces and varies in intensity with the severity of the hepatic lesion. In the earliest stages polynuclear leucocytes and cells of lymphoid type are numerous; at the end of four days elongated cells resembling fibroblasts may be numerous. Foci of necrotic cells in contact with a portal space are occasionally found in process of invasion and re-

placement by the cells which have accumulated. In correspondence with the severity of the hepatic lesion accumulation of cells in and about the portal tissue has been more conspicuous in animals which have received meat. In one animal which lived eleven days fibroblasts are numerous in the portal tissue and form outshoots into the adjacent hepatic parenchyma, whereas within the lobules occur several foci in which young fibroblasts replace hepatic cells. Comparison with earlier lesions suggests that young connective tissue has taken the place of liver cells which have undergone necrosis and disintegration.

The effects of diet upon the toxicity of phosphorus differ from those observed with chloroform. Whereas fat has uniformly increased the toxicity of chloroform, meat has with equal constancy increased susceptibility to phosphorus. There has been no noteworthy difference in the toxicity of phosphorus with carbohydrate and with fatty diet. The experiments suggest no obvious explanation of these differences in the effects of diet upon chloroform and phosphorus. Certain differences between chloroform and phosphorus may be cited. Chloroform produces necrosis of the center of each hepatic lobule, whereas phosphorus usually produces widespread fatty degeneration, which is more intense in the periphery of the lobule. Phosphorus is less destructive to the hepatic cell, but occasionally, when given in large dose, and almost constantly, when given to animals which have received a diet of meat, causes widespread necrosis localized especially in the periphery of the lobule. Both substances are soluble in fat, but chloroform is absorbed more readily and its toxic action on the liver is far more quickly exhibited. It is evident from the experiments that the fatty content of the liver has little influence upon the severity of the lesion which follows the slow deposition of phosphorus in the liver.

#### ALCOHOL.

Alcohol has been selected for the following experiments because it is believed to be a common cause of cirrhosis of the liver in man. Its toxicity was first tested upon white rats receiving a mixed diet consisting of oats and other substances. In this preliminary test 0.25 of a cubic centimeter of absolute alcohol per 100 grams of body-

weight, diluted with an equal part of water and injected subcutaneously, failed to kill, whereas doses of 0.5 and 0.7 of a cubic centimeter, and 1 cubic centimeter were fatal. Large quantities of alcohol produced anesthesia from which the animal did not recover.

TABLE X.

*Alcohol.*

Diet.	Weight before diet.	Weight after 5 dys.	Dose per 100 gm.	Length of life.	Remarks.
Oats and sugar	149 gm.	137 gm.	0.2 c.c.	*	Slight fatty degeneration of liver.
	160 gm.	145 gm.	0.35 c.c.	*	
	153 gm.	152 gm.	0.5 c.c.	*	
	167 gm.	155 gm.	0.65 c.c.	*	
	169 gm.	164 gm.	0.8 c.c.	1 dy.	
Meat	127 gm.	122 gm.	0.2 c.c.	*	No noteworthy lesion found.
	153 gm.	143 gm.	0.35 c.c.	*	
	155 gm.	147 gm.	0.5 c.c.	*	
	167 gm.	150 gm.	0.65 c.c.	*	
	170 gm.	165 gm.	0.8 c.c.	1 dy.	
Fat	118 gm.	116 gm.	0.2 c.c.	*	
	160 gm.	147 gm.	0.35 c.c.	*	
	157 gm.	151 gm.	0.5 c.c.	*	
	165 gm.	160 gm.	0.65 c.c.	*	
	167 gm.	164 gm.	0.8 c.c.	*	

\* = lived.

TABLE XI.

*Alcohol.*

Diet.	Weight before diet.	Weight after 6 dys.	Dose per 100 gm.	Length of life.	Remarks.
Meat	130 gm.	135 gm.	0.15 c.c.	3 dys.	No noteworthy lesion found.
	133 gm.	136 gm.	0.3 c.c.	*	No noteworthy lesion found.
	127 gm.	145 gm.	0.45 c.c.	4 dys.	
	140 gm.	145 gm.	0.6 c.c.	*	
	157 gm.	162 gm.	0.75 c.c.	*	
Oats and sugar	125 gm.	127 gm.	0.15 c.c.	*	
	132 gm.	138 gm.	0.3 c.c.	*	
	140 gm.	147 gm.	0.45 c.c.	*	
	143 gm.	148 gm.	0.6 c.c.	*	
	139 gm.	152 gm.	0.75 c.c.	*	
Fat	127 gm.	132 gm.	0.15 c.c.	*	No noteworthy lesion found.
	132 gm.	145 gm.	0.3 c.c.	*	
	135 gm.	145 gm.	0.45 c.c.	*	
	152 gm.	151 gm.	0.6 c.c.	*	
	140 gm.	156 gm.	0.75 c.c.	1 dy.	

\* = lived.

The preceding experiments were performed under conditions similar to those present in the tests with chloroform and phosphorus. The animals were on special diets during five days (table X).

In the next series of experiments animals received special diets during six days (table XI).

Although the experiments show that the maximum quantities of alcohol administered were sufficient to cause death they furnish no proof that the toxicity of alcohol can be influenced by the diets employed. There is no evidence that alcohol, like chloroform, causes necrosis of the liver.



# THE INFLUENCE OF DIET UPON NECROSIS CAUSED BY HEPATIC AND RENAL POISONS.

## PART II. DIET AND THE NEPHRITIS CAUSED BY POTASSIUM CHROMATE, URANIUM NITRATE, OR CHLOROFORM.\*

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Since the experiments which have been recorded demonstrate that lesions of the liver produced by chloroform and phosphorus may be profoundly influenced by diet, experiments have been performed to determine if substances which produce necrosis of the parenchymatous cells of the kidney exhibit similar relations to diet. Potassium chromate and uranium nitrate have been chosen because they have a selective action on the kidney causing necrosis of the renal tubules and leaving the parenchymatous cells of the liver and other organs relatively unaffected. These substances have the further advantage that they have been much used to produce acute nephritis in animals. They cause albuminuria, and in the white rat casts are very numerous within the tubules of the kidney.

### THE EFFECT OF DIET UPON THE KIDNEY.

White rats were given during five and eight days (a) oats and sugar, (b) meat alone, or (c) fat alone, and the amount of visible fat within the kidney was determined by staining with Sudan III. In all kidneys examined the stain gives to the tubules of the cortex and of the subcortical zone containing the loops of Henle a pinkish color, but in animals which have received the carbohydrate diet fat droplets are almost wholly absent. Groups of fine, brightly stained fat droplets were occasionally found in a convoluted tubule. In animals which received meat fat was somewhat more abundant. The kidney of one animal which had received meat

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contained a moderate amount of visible fat present in tubules within the medullary striæ, in convoluted tubules situated in the part of the cortex in contact with the medulla, and occasionally in the loops of Henle extending into the medulla. This animal had bronchopneumonia with multiple abscesses in the lungs. The kidneys of animals which had received a diet of fat contained fat in fair abundance. Many of the tubules within the medullary rays of the cortex and many of the loops of Henle in the medulla immediately below the cortex contained conspicuously stained coarse fat droplets so abundant that the affected tubules were sharply outlined. In the convoluted tubules there was little fat save in the lowermost part of the cortex; here groups of tubules contain fat droplets closely crowded together. The distribution of fat indicates that the fatty diet may cause accumulation of fat in the cells of the loops of Henle.

#### POTASSIUM CHROMATE.

Potassium chromate in 2.5 per cent. aqueous solution has been injected subcutaneously in quantities varying from 0.05 to 0.3 of a cubic centimeter (0.00125 to 0.0075 of a gram). In the following series of experiments animals were given during nineteen days diets consisting of (a) oats and sugar, (b) pig's heart, and (c) beef fat. The special diets were continued after administration of potassium chromate (table I).

In the following series of experiments diets of meat and fat administered during an unusually long period have been accompanied by considerable loss of weight (for meat an average of 22 grams,

TABLE I.  
*Potassium Chromate.*

Diet.	Weight before diet.	Weight after 19 dys.	Dose per 100 gm.	Length of life.	Remarks.
Oats and sugar	125 gm.	143 gm.	0.1 c.c.	*	
	158 gm.	148 gm.	0.15 c.c.	5 dys.	Necrosis $\frac{2}{3}$ convoluted tubules; calcification.
	148 gm.	152 gm.	0.2 c.c.	8 dys.	Necrosis $\frac{1}{2}$ convoluted tubules; calcification.
	154 gm.	155 gm.	0.25 c.c.	1 dy.	Beginning necrosis of convoluted tubules.
	155 gm.	172 gm.	0.3 c.c.	3 dys.	Necrosis $\frac{1}{3}$ convoluted tubules.

Average length of survival  
with carbohydrate diet. . . . .  $4\frac{1}{4}$  + dys.

TABLE I.—*Concluded.*

Diet.	Weight before diet.	Weight after 19 dys.	Dose per 100 gm.	Length of Life.	Remarks.
Meat	125 gm.	100 gm.	0.1 c.c.	3 dys.	Casts in kidney.
	155 gm.	128 gm.	0.15 c.c.	3 dys.	Necrosis $\frac{1}{2}$ convoluted tubules.
	157 gm.	132 gm.	0.2 c.c.	1 dy.	Casts in kidney.
	153 gm.	134 gm.	0.25 c.c.	2 dys.	Necrosis $\frac{1}{2}$ convoluted tubules.
	160 gm.	142 gm.	0.3 c.c.	1 dy.	Beginning necrosis of convoluted tubules.
Average length of survival with meat diet. . . . . 2 dys.					
Fat	145 gm.	109 gm.	0.1 c.c.	6 dys.	Necrosis $\frac{1}{2}$ convoluted tubules; calcification.
	135 gm.	110 gm.	0.15 c.c.	3 dys.	Necrosis $\frac{3}{4}$ convoluted tubules.
	151 gm.	121 gm.	0.2 c.c.	3 dys.	Necrosis $\frac{2}{3}$ convoluted tubules.
	155 gm.	127 gm.	0.25 c.c.	2 dys.	Beginning necrosis of convoluted tubules.
	160 gm.	133 gm.	0.3 c.c.	1 dy.	No lesion found.
Average length of survival with fat diet. . . . . 3 dys.					

\* = lived.

TABLE II.

*Potassium Chromate.*

Diet.	Weight before diet.	Weight after 6 dys.	Dose per 100 gm.	Length of life.	Remarks.
Oats and sugar	72 gm.	77 gm.	0.05 c.c.	*	
	67 gm.	77 gm.	0.075 c.c.	4 dys.	Necrosis $\frac{1}{2}$ convoluted tubules.
	87 gm.	100 gm.	0.1 c.c.	3 dys.	Necrosis $\frac{1}{2}$ convoluted tubules.
	200 gm.	205 gm.	0.125 c.c.	5 dys.	Necrosis $\frac{1}{2}$ convoluted tubules.
	200 gm.	205 gm.	0.15 c.c.	5 dys.	Necrosis $\frac{2}{3}$ convoluted tubules.
Average length of survival with carbohydrate diet. . . . . $4\frac{1}{4}$ + dys.					
Meat and oats	67 gm.	80 gm.	0.05 c.c.	*	
	85 gm.	97 gm.	0.075 c.c.	3 dys.	Necrosis $\frac{1}{2}$ convoluted tubules.
	80 gm.	108 gm.	0.1 c.c.	4 dys.	Necrosis $\frac{2}{3}$ convoluted tubules; calcification.
	198 gm.	102 gm.	0.125 c.c.	3 dys.	Necrosis $\frac{3}{4}$ convoluted tubules.
	262 gm.	270 gm.	0.15 c.c.	3 dys.	Necrosis $\frac{3}{4}$ convoluted tubules.
Average length of survival with meat diet. . . . . $3\frac{1}{4}$ + dys.					
Fat and oats	65 gm.	60 gm.	0.05 c.c.	*	
	70 gm.	75 gm.	0.075 c.c.	3 dys.	Necrosis $\frac{1}{2}$ convoluted tubules.
	120 gm.	125 gm.	0.1 c.c.	3 dys.	Necrosis $\frac{1}{2}$ convoluted tubules.
	155 gm.	150 gm.	0.125 c.c.	3 dys.	Necrosis $\frac{2}{3}$ convoluted tubules.
	275 gm.	275 gm.	0.15 c.c.	5 dys.	Necrosis $\frac{2}{3}$ convoluted tubules.
Average length of survival with fat diet. . . . . $3\frac{1}{2}$ + dys.					

\* = lived.

for fat, 27.2 grams), whereas animals which received oats and sugar gained in weight (an average of 6 grams). To avoid the disturbance of nutrition produced by prolonged feeding with meat or fat, animals have been fed in the preceding series of experiments (table II) during six days with (a) oats and cane sugar, (b) beefsteak and rolled oats, and (c) fat inseparably mixed with rolled oats. Animals which received oats and sugar and those which received meat and oats gained in weight; those with oats and fat maintained their weight.

TABLE III.  
*Potassium Chromate.*

Diet.	Weight before diet.	Weight after diet.	Dose per 100 gm.	Length of life.	Remarks.
Oats and sugar	71 gm.	67 gm.	0.05 c.c.	*	Necrosis $\frac{1}{3}$ convoluted tubules.
	75 gm.	68 gm.	0.1 c.c.	*	
	80 gm.	72 gm.	0.15 c.c.	*	
	88 gm.	82 gm.	0.2 c.c.	*	
	94 gm.	85 gm.	0.25 c.c.	5 dys.	
Average length of survival with carbohydrate diet. . . . . 5 + dys.					
Egg-yolk	82 gm.	82 gm.	0.05 c.c.	*	Necrosis $\frac{1}{3}$ convoluted tubules; calcification. Necrosis $\frac{3}{4}$ convoluted tubules; calcification.
	77 gm.	84 gm.	0.1 c.c.	*	
	84 gm.	94 gm.	0.15 c.c.	*	
	93 gm.	97 gm.	0.2 c.c.	6 dys.	
	93 gm.	100 gm.	0.25 c.c.	3 dys.	
Average length of survival with egg diet. . . . . 4 $\frac{1}{2}$ + dys.					
Meat	63 gm.	60 gm.	0.05 c.c.	2 dys.	No lesion of kidney found. No lesion of kidney found. Necrosis $\frac{1}{2}$ convoluted tubules; calcification. Necrosis $\frac{1}{2}$ convoluted tubules. Necrosis $\frac{2}{3}$ convoluted tubules.
	78 gm.	74 gm.	0.1 c.c.	2 dys.	
	82 gm.	82 gm.	0.15 c.c.	4 dys.	
	85 gm.	92 gm.	0.2 c.c.	5 dys.	
	95 gm.	104 gm.	0.25 c.c.	3 dys.	
Average length of survival with meat diet. . . . . 3 $\frac{1}{3}$ dys.					

\* = lived.

In the next series of experiments (table III) animals have been given during six days diets consisting of (a) oats and cane sugar, (b) beefsteak, and (c) boiled egg-yolk which contains 35 per cent. of fat including cholesterin and lecithin. After injection of potas-

sium chromate animals on diets of meat and of egg-yolk were allowed to eat freely of rolled oats.

Animals which received oats and sugar lost in weight, the average loss being 6.8 grams, whereas those on meat gained an average of 1.8 grams, and those on egg-yolk, 5.6 grams. The last three series of experiments fully confirm the view that the effect of diet upon the toxicity of the substances which have been tested bears no constant relation to the body-weight and is not the result of partial inanition.

In the three series of experiments with potassium chromate the number of animals which have survived and the average length of life of those which have died uniformly demonstrate the protective influence of a carbohydrate diet. By combining oats with meat and with fat (table II) differences between the three diets have been reduced to a minimum but still persist. The protective action of the carbohydrate diet is referable to the period preceding the production of nephritis by potassium chromate, for this protection is evident even though carbohydrate has been given to all animals after the poison has been administered.

Animals which have received meat have shown the greatest susceptibility to the action of potassium chromate, but there has been little difference between the effect of protein and of fat. Animals which have received egg-yolk which contains cholesterin in abundance have not exhibited remarkable susceptibility to potassium chromate.

The changes produced in the kidney of the white rat by potassium chromate have been studied carefully in order to define their relation to the lesions of the liver produced by chloroform and phosphorus and to compare the severity of the lesions in animals which have been given different diets. Necrosis is limited to the convoluted tubules within the cortex of the kidney; there is no necrosis of the liver. In animals which have died within one day after administration of potassium chromate no changes may be found in the kidney; within one or two days hyaline casts are usually found in the straight tubules and a few of the convoluted tubules may exhibit necrosis; the cells are swollen and occasionally hyaline and the nuclei have disappeared or remain as contracted, deeply stained bodies.

At the end of three days the extent of necrosis is well defined, and with large doses of potassium chromate almost every convoluted tubule of the kidney is a swollen hyaline column without nuclei; the endothelial cells of the capillaries persist, and the glomeruli, the medullary rays of the cortex, and the loops of Henle in the medulla just below the cortex are intact. An attempt has been made to estimate roughly the extent of necrosis in animals which have survived three or more days. When the kidneys of animals which have lived three or more days are compared, necrosis is usually found to be somewhat more extensive in animals which have received meat or fat than in animals on a carbohydrate diet. In table II, in which all animals lived three days, necrosis is most severe in animals with meat, less with fat, and least with carbohydrate. In animals which have lived four or more days calcium is often deposited in the necrotic tubules; there is apparently no relation between diet and the occurrence of calcification.

#### URANIUM NITRATE.

Uranium nitrate, like potassium chromate, causes necrosis of the renal tubules. The physiological studies of Schlayer and Hedinger,<sup>1</sup> Pearce,<sup>2</sup> and others have shown that reactions referable to the vascular apparatus of the kidney are not impaired by these substances; whereas with the nephritis produced, for example, by cantharidin or arsenic, the volume of the kidney does not diminish under the influence of sensory stimuli or adrenalin; while diuretics, such as caffeine or 5 per cent. salt solution, do not dilate the blood vessels and fail to produce diuresis. Uranium nitrate exhibits peculiarities not observed with potassium chromate. At a certain stage of intoxication with uranium nitrate, diuretics, such as 5 per cent. salt solution, urea, dextrose, etc., cause dilatation of the blood vessels of the kidney but no increased flow of urine.<sup>3</sup> Well known observations of Richter<sup>4</sup> have demonstrated that uranium nitrate given to rabbits causes subcutaneous edema and accumulation of fluid in the serous cavities when hydremic plethora is produced by the daily administra-

<sup>1</sup> Schlayer and Hedinger, *Deutsch. Arch. f. klin. Med.*, 1907, xc, 1.

<sup>2</sup> Pearce, R. M., *Arch. Int. Med.*, 1909, iii, 422.

<sup>3</sup> Schlayer, Hedinger, and Takayasu, *Deutsch. Arch. f. klin. Med.*, 1907, xci, 59.

<sup>4</sup> Richter, P. F., *Beitr. z. klin. Med., Festschr. f. Senator*, 1904, 283; *Berl. klin. Wchnschr.*, 1905, xlii, 384.

tion of water by stomach. Edema is not similarly caused by potassium chromate. A further difference between the action of the two substances has been noted by Christian and O'Hare<sup>5</sup> who found that uranium nitrate causes a lesion of the glomeruli characterized by the presence of hyaline droplets in the capillary loops and by other changes.

Uranium nitrate has been administered in 0.5 per cent. aqueous solution by subcutaneous injection. In the following series of experiments (table IV) doses varying from 0.05 to 0.3 of a cubic centimeter (0.00025 to 0.0015 of a gram) have been given to animals which during four days had received (a) oats and cane sugar, (b) beefsteak, or (c) beef fat; the diets were continued six days after injection of uranium nitrate.

TABLE IV.  
*Uranium Nitrate.*

Diet.	Weight before diet.	Weight after 4 dys.	Dose per 100 gm.	Length of life.	Remarks.
Oats and sugar	95 gm.	106 gm.	0.05 c.c.	*	
	119 gm.	110 gm.	0.1 c.c.	6 dys.	Kidney: necrosis ( $\frac{1}{2}$ ).
	138 gm.	140 gm.	0.15 c.c.	7 dys.	Kidney: scant necrosis; calcification.
	150 gm.	142 gm.	0.2 c.c.	9 dys.	Kidney: necrosis ( $\frac{1}{3}$ ).
	132 gm.	149 gm.	0.25 c.c.	*	Kidney: necrosis ( $\frac{1}{3}$ ); calcification.
	165 gm.	172 gm.	0.3 c.c.	10 dys.	
Average length of survival with carbohydrate diet.....8+dys.					
Meat	85 gm.	88 gm.	0.05 c.c.	*	
	95 gm.	93 gm.	0.1 c.c.	*	
	127 gm.	127 gm.	0.15 c.c.	7 dys.	Kidney: scant necrosis.
	127 gm.	132 gm.	0.2 c.c.	4 dys.	Kidney: necrosis ( $\frac{1}{4}$ ).
	145 gm.	135 gm.	0.25 c.c.	4 dys.	Kidney: necrosis ( $\frac{1}{4}$ ); calcification.
	280 gm.	267 gm.	0.3 c.c.	8 dys.	Kidney: necrosis ( $\frac{1}{5}$ ); calcification.
Average length of survival with meat diet.....5 $\frac{3}{4}$ dys.					
Fat	85 gm.	78 gm.	0.05 c.c.	10 dys.	Kidney: necrosis ( $\frac{1}{5}$ ); calcification.
	96 gm.	96 gm.	0.1 c.c.	8 dys.	Kidney: scant necrosis; calcification.
	128 gm.	114 gm.	0.15 c.c.	6 dys.	Kidney: necrosis ( $\frac{2}{3}$ ); calcification.
	140 gm.	130 gm.	0.2 c.c.	7 dys.	Kidney: necrosis ( $\frac{2}{3}$ ); calcification.
	145 gm.	135 gm.	0.25 c.c.	6 dys.	Kidney: necrosis ( $\frac{2}{3}$ ).
	230 gm.	225 gm.	0.3 c.c.	9 dys.	Kidney: necrosis ( $\frac{2}{3}$ ); calcification.
Average length of survival with fat diet.....7 $\frac{2}{3}$ dys.					

\* = lived.

<sup>5</sup> Christian, H. A., and O'Hare, J. P., *Jour. Med. Research*, 1913, xxviii, 227.

In this series of experiments animals upon a diet of fat have shown greatest susceptibility to uranium nitrate. The number of animals which survived has been the same on diets of meat and of carbohydrate, but the average duration of life has been considerably longer after a carbohydrate diet. These differences have been almost obliterated when oats have been given with meat and fat. In the following series of experiments (table V) oats were combined with meat and fat during three days; during the next three days animals received meat alone and fat alone and after administration of uranium nitrate all animals received oats. Since animals were allowed to eat oats freely, it is probable that the special diets were effective only during three days.

TABLE V.  
*Uranium Nitrate.*

Diet.	Weight before diet.	Weight after 6 dys.	Dose per 100 gm.	Length of life.	Remarks.
Oats and sugar	120 gm.	110 gm.	0.05 c.c.	10 dys.	Kidney: necrosis ( $\frac{1}{2}$ ); calcification.
	132 gm.	133 gm.	0.1 c.c.	9 dys.	Kidney: necrosis ( $\frac{1}{3}$ ); calcification.
	145 gm.	143 gm.	0.15 c.c.	9 dys.	Kidney: necrosis ( $\frac{1}{3}$ ).
	145 gm.	143 gm.	0.2 c.c.	7 dys.	Kidney: necrosis ( $\frac{1}{3}$ ); calcification.
	160 gm.	170 gm.	0.25 c.c.	6 dys.	Kidney: scant necrosis; calcification.
Average length of survival with carbohydrate diet . . . . . $8\frac{1}{2}$ dys.					
Meat	117 gm.	122 gm.	0.05 c.c.	*	
	145 gm.	127 gm.	0.1 c.c.	6 dys.	Kidney: necrosis ( $\frac{1}{3}$ ).
	150 gm.	133 gm.	0.15 c.c.	7 dys.	Kidney: necrosis ( $\frac{1}{3}$ ); calcification.
	140 gm.	160 gm.	0.2 c.c.	10 dys.	Kidney: necrosis ( $\frac{1}{4}$ ); calcification.
	177 gm.	162 gm.	0.25 c.c.	6 dys.	Kidney: scant necrosis; calcification.
Average length of survival with meat diet . . . . . $7\frac{1}{4}$ + dys.					
Fat	125 gm.	115 gm.	0.05 c.c.	8 dys.	Kidney: necrosis ( $\frac{2}{3}$ ); calcification.
	135 gm.	128 gm.	0.1 c.c.	8 dys.	Kidney: necrosis ( $\frac{2}{3}$ ); calcification.
	145 gm.	148 gm.	0.15 c.c.	7 dys.	Kidney: necrosis ( $\frac{1}{2}$ ).
	156 gm.	159 gm.	0.2 c.c.	8 dys.	Kidney: necrosis ( $\frac{1}{2}$ ); calcification.
	157 gm.	160 gm.	0.25 c.c.	7 dys.	Kidney: necrosis ( $\frac{1}{3}$ ); calcification.
Average length of survival with fat diet . . . . . $7\frac{2}{3}$ dys.					

\* = lived.

In the foregoing series of experiments the diets which have been employed have been associated with no noteworthy differences in



the number of animals which died or in the average duration of life. A study of changes in the kidneys has shown that the more advanced necrosis occurs in animals which have received a diet of fat.

Since nearly all animals in the two last series of experiments have died, it has seemed probable that differences might be more accurately tested by smaller doses. In the next series of experiments (table VI) special diets have been administered during five days; from 0.02 to 0.1 of a cubic centimeter of a 0.5 per cent. solution (0.0001 to 0.0005 of a gram) of uranium nitrate has been injected subcutaneously and after its administration oats have been given to all animals.

TABLE VI.  
*Uranium Nitrate.*

Diet.	Weight before diet.	Weight after 5 dys.	Dose per 100 gm.	Length of life.	Remarks.
Oats and sugar	138 gm.	137 gm.	0.02 c.c.	*	Kidney not examined.
	165 gm.	140 gm.	0.04 c.c.	*	
	155 gm.	154 gm.	0.06 c.c.	7 dys.	
	168 gm.	159 gm.	0.08 c.c.	*	
	260 gm.	267 gm.	0.1 c.c.	*	
Average duration of life with carbohydrate diet.....7+dys.					
Meat	142 gm.	130 gm.	0.02 c.c.	9 dys.	Kidney: scant necrosis.
	138 gm.	132 gm.	0.04 c.c.	*	
	150 gm.	137 gm.	0.06 c.c.	7 dys.	Kidney: scant necrosis.
	164 gm.	162 gm.	0.08 c.c.	10 dys.	Kidney: not examined.
	185 gm.	172 gm.	0.1 cc.	11 dys.	Kidney: scant necrosis.
Average duration of life with meat diet.....9 $\frac{1}{4}$ +dys.					
Fat	115 gm.	99 gm.	0.02 c.c.	7 dys.	Kidney: necrosis ( $\frac{1}{4}$ ).
	130 gm.	115 gm.	0.04 c.c.	*	
	142 gm.	127 gm.	0.06 c.c.	9 dys.	Kidney: necrosis ( $\frac{1}{4}$ ).
	155 gm.	140 gm.	0.08 c.c.	11 dys.	Kidney: necrosis ( $\frac{1}{4}$ ).
	290 gm.	280 gm.	0.1 c.c.	7 dys.	Kidney: necrosis ( $\frac{3}{4}$ ).
Average duration of life with fat diet.....8 $\frac{1}{2}$ +dys.					

\* = lived.

The experiments with uranium nitrate demonstrate that carbohydrate exerts a protective action. A survey of the three last tables shows that only one of six animals on carbohydrate diet receiving

less than 0.1 of a cubic centimeter died, only one of four on meat diet receiving less than 0.06 of a cubic centimeter died, and only one of two animals on a fat diet receiving less than 0.05 of a cubic centimeter died, a fatal result being almost constant after the respective diets with doses above those named. Susceptibility to uranium nitrate after a diet of meat and after a diet of fat is almost the same; susceptibility is somewhat greater after fat.

Since the object of these experiments upon the kidney has been to determine if diet influences the incidence of necrosis, a careful study of the kidney has been made. The liver and other organs show no change comparable to that which occurs in the kidney. It may be assumed that animals which survive have suffered less profound renal changes than those which die. In the two series recorded in tables IV and V, relatively large doses having been employed, nearly all animals died after an interval which does not vary very greatly. These series offer an opportunity to determine if the extent of the lesion is influenced by diet.

Although uranium nitrate causes necrosis of the renal tubules, the lesion is not identical with that of potassium chromate. Whereas potassium chromate causes death within from one to five days uranium nitrate has rarely caused death before the end of six days. With small doses of uranium nitrate the convoluted tubules are almost wholly unaffected; in the lowermost part of the cortex in immediate contact with the medulla small groups of convoluted tubules have lost their nuclei and become hyaline. In the medullary striæ of the cortex necrosis has usually occurred in many tubules. In the medulla just below the level of the glomeruli tubules which have undergone coagulative necrosis are abundant and have the characters of the loops of Henle, which here dip into the medulla. With more advanced lesions the greater part of all tubules within both the cortical striæ and subcortical zone may be necrotic, whereas the convoluted tubules are little changed. With increasingly severe lesions, such as occur after a fat diet, the convoluted tubules are implicated and a considerable proportion of the cortex undergoes necrosis. Calcium salts are quickly deposited in the dead tissue and, stained with hematoxylin, define very sharply the chief localization

of the lesion in the medullary striæ and the zone just beneath the cortex.

The extent of necrosis is considerably greater in animals which have received fat than in those on other diets, but no noteworthy difference has been found between the kidneys of those with meat and those with carbohydrate. In tables IV, V, and VI a rough estimate has been made of the relation of the necrotic tissue to the total area (cortex plus subcortical zone) in which necrosis occurs. In the tables this estimate is expressed by a fraction in parenthesis. After a diet of fat from two thirds to three fourths of this area may be implicated (tables IV and V), whereas with carbohydrate or meat the necrotic part is usually not more than from one fifth to one third when the corresponding doses of uranium nitrate are employed.

It is noteworthy that necrosis of the renal tubules with uranium nitrate is most severe after a diet of fat and occurs in those situations in which fat has been found in the normal kidney of animals fed upon fat, namely, in the medullary striæ, in the lowermost part of the cortex, and in the medulla immediately below the cortex. The loops of Henle of a rat fed upon fat become unusually susceptible to the action of uranium nitrate.

#### CHLOROFORM.

The occurrence of necrosis caused by uranium nitrate in parts of the kidney in which fatty infiltration may occur has suggested the possibility that a substance such as chloroform, which is soluble in fat, may produce necrosis in the same situations, for it has been shown that animals which have received fat are especially susceptible to the toxic action of chloroform. Furthermore, fat is deposited in the center of the hepatic lobule of white rats which have received a fat diet and the necrosis of chloroform occurs in this part of the lobule. Renal lesions following the administration of chloroform have been noted in some of the experiments<sup>6</sup> which have been described. When chloroform produces a fatal lesion of the liver death occurs within three or four days. Animals which sur-

<sup>6</sup> Opie, E. L., and Alford, L. B., *Jour. Exper. Med.*, 1915, xxi, 1, tables I, II, and VI.

vive this period may die after from six to eleven days with evident lesions of the kidney. The organ is enlarged and bright yellow. There is fatty degeneration or both necrosis and fatty degeneration of the renal tubules, and casts are abundant (page 6). In animals which have died with hepatic lesions within three or four days, well defined lesions of the kidney are often present.

In table VII changes found in the kidneys of animals which died after various periods following the administration of chloroform are described. These animals are some of those concerning which data are recorded in table VI of the preceding paper.<sup>7</sup>

TABLE VII.

*Chloroform.*

Diet.	Dose per 100 gm.	Length of life.	Kidney.
Fat	0.025 c.c.	8 dys.	Necrosis ( $\frac{1}{2}$ ) of medullary striæ, of convoluted tubules in lowermost half of cortex, and of loops of Henle in adjacent medulla; slight calcification.
Fat	0.075 c.c.	3 dys.	Casts in tubules.
Fat	0.125 c.c.	6 dys.	Necrosis ( $\frac{1}{2}$ ) in situations named above.
Fat	0.225 c.c.	4 dys.	Fatty degeneration diffusely distributed; albumin in glomerular capsules; casts.
Brain	0.025 c.c.	2 dys.	No lesion found.
Brain	0.075 c.c.	2 dys.	Fatty degeneration diffusely distributed; albumin in glomerular capsules; casts.
Brain	0.125 c.c.	2 dys.	Fatty degeneration most advanced in medullary striæ and subcortical zone.
Brain	0.225 c.c.	4 dys.	Necrosis almost wholly limited to medullary striæ with beginning calcification; fatty degeneration.

The renal lesion which is produced by chloroform is inconspicuous in animals which have lived less than four days. Necrosis of renal tubules occurs, and although it may affect tubules in any part of the cortex it is most severe in the medullary striæ, in the lowermost part of the cortex, and in the immediately adjacent subcortical part of the medulla. Fat infiltration after a diet rich in fat has been found in the same situations and is much less advanced in other parts of the cortex. Chloroform which is readily soluble in fat produces necrosis in those parts of both liver and kidney in which visible fat accumulates.

<sup>7</sup> Opie, E. L., and Alford, L. B., *loc. cit.*

## DISCUSSION.

The effect of diet upon the toxicity of two substances, namely, chloroform and phosphorus, capable of causing necrosis of the liver, has been studied, and a similar investigation of two substances which cause necrosis of renal tubules, namely, potassium chromate and uranium nitrate, has been made. All of these substances are more toxic for animals which have received a diet of meat than for those which have received a diet consisting in great part of carbohydrate (oats and cane sugar). Upon these diets white rats maintain their weight and remain in good health. Study of the histological changes in animals which have died shows that susceptibility to these poisons is dependent upon the varying extent of necrosis in the liver or kidney induced by diet.

The protective action of carbohydrates, preventing destruction of hepatic or renal cells, is in accord with observations which have shown that a carbohydrate diet prevents the disintegration of body protein. Voit, Rubner, and others<sup>8</sup> have shown that carbohydrate given to a healthy individual diminishes the amount of protein needed to maintain nitrogen equilibrium and in starvation reduces the elimination of nitrogen. It is well known that the fever of acute infections is accompanied by increased excretion of nitrogen in the urine due to augmented activity of protein catabolism. Fritz Voit<sup>9</sup> and Linser and Schmidt<sup>10</sup> found that elevation of temperature caused by immersion in warm water was accompanied by increased elimination of nitrogen; they found that it was possible to retard this increased protein catabolism by adding carbohydrate to the food. Shaffer and Coleman<sup>11</sup> have shown that the loss of body protein which occurs with typhoid fever may be retarded by the use of diets of high caloric value especially rich in carbohydrate, and, if the supply of carbohydrate is sufficient, the loss of nitrogen derived from body protein may be prevented. Coincident diminution of the excretion of creatin and of total sulphur indicates that febrile de-

<sup>8</sup> For literature on the subject see Lusk, G., *The Elements of the Science of Nutrition*, Philadelphia and London, 1909.

<sup>9</sup> Voit, F., *Sitzungsber. d. Gesellsch. f. Morphol. u. Physiol. in München*, 1895, xi, 120; cited by Shaffer, P. A., and Coleman, W., *Arch. Int. Med.*, 1909, iv, 538.

<sup>10</sup> Linser, P., and Schmidt, J., *Deutsch. Arch. f. klin. Med.*, 1904, lxxix, 514.

<sup>11</sup> Shaffer, P. A., and Coleman, W., *loc. cit.*

struction of body protein is prevented. What relation disintegration of body protein bears to parenchymatous degeneration of the organs, focal necrosis of the liver and spleen, and necrosis affecting the specific lesions of typhoid fever cannot be defined with our present knowledge of the subject.

The urine furnishes similar evidence of protein disintegration after administration of chloroform. Strassmann<sup>12</sup> and other observers have shown that anesthesia produced by inhalation of chloroform is followed by increased elimination of urinary nitrogen, sulphur, and phosphoric acid. The experimental studies of Howland and Richards<sup>13</sup> have demonstrated that the increased elimination of nitrogen and sulphur which accompanies intense disintegration of body protein occurs in association with necrosis of the liver, which is the characteristic lesion of delayed chloroform poisoning in all mammals. A similar increase of protein catabolism occurs as the result of phosphorus poisoning (Ray, McDermott, and Lusk<sup>14</sup>).

Necrosis produced by chloroform, phosphorus, and similar substances is perhaps the anatomical expression of advanced disintegration of body protein. Carbohydrate may tend to limit this necrosis by protecting body protein. Protein diet, by virtue of the specific dynamic action of protein diet demonstrated by Rubner,<sup>15</sup> increases the activity of metabolism, indicated by increased heat formation.

It is noteworthy that the quantity of carbohydrate needed to maintain nitrogen equilibrium may be increased by disease of the liver. Tallqvist<sup>16</sup> found that increased quantity of carbohydrate is required with catarrhal jaundice, cholelithiasis with jaundice, cirrhosis, and certain other hepatic diseases. He believes that the change occurs because the liver is no longer able to store glycogen effectively.

The toxicity of two substances, namely, chloroform and uranium nitrate, has been greater after a diet of fat than after diets of meat or of carbohydrate. Necrosis caused by chloroform occurs in the

<sup>12</sup> Strassman, F., *Virchows Arch. f. path. Anat.*, 1889, cxv, 1.

<sup>13</sup> Howland, J., and Richards, A. N., *Jour. Exper. Med.*, 1909, xi, 344.

<sup>14</sup> Ray, W. E., McDermott, T. S., and Lusk, G., *Am. Jour. Physiol.*, 1899, iii, 139.

<sup>15</sup> Rubner, M., *Die Gesetze des Energieverbrauchs*, Leipzig, 1902.

<sup>16</sup> Tallqvist, T. W., *Arch. f. Hyg.*, 1908, lxx, 39.

centers of the hepatic lobules and in the loops of Henle. Infiltration with fat occurs in these situations when white rats are fed with fat. The ready solubility of chloroform in fat suggests the probability that necrosis occurs because chloroform is fixed by the fat present in the cell. The susceptibility of the cells of the kidney and of the liver to necrosis has a part in the localization of the lesion, for all cells of the body which contain fat do not undergo necrosis. Meyer and Overton maintain that narcosis occurs because a substance soluble in lipoids accumulates in the cells of the central nervous system. They believe that the efficiency of an anesthetic is dependent upon the readiness with which it passes from an aqueous solution into lipid and may be conveniently expressed by its coefficient of distribution in oil and water. It is probable that the occurrence of necrosis caused by chloroform is dependent upon two factors: (1) the coefficient of distribution in fat and water causing the fixation of chloroform, and (2) the injurious action of chloroform upon the protoplasm of the cell, measurable in part by increased activity of protein catabolism.

Of the two substances which cause necrosis of renal tubules, uranium nitrate exhibits maximum toxicity in animals which have received fat, whereas with potassium chromate the susceptibility of animals which have received meat is somewhat greater than the susceptibility of those which have received fat. It is noteworthy that the lesion of uranium nitrate is severest in those tubules (loops of Henle) which accumulate visible fat when the animal is fed upon fat.

There is a close analogy between certain human lesions and those produced by the substances which have been employed. Toxemia of pregnancy, acute yellow atrophy of the liver, and certain bacterial infections<sup>17</sup> are characterized by necrosis of parenchymatous cells of the liver and not infrequently of the kidney, resembling very closely the changes produced by chloroform. In eclampsia the distribution of hepatic necrosis is similar to that which has been caused by phosphorus. Focal necrosis of parenchymatous cells occurs in the liver, spleen, and other organs during typhoid fever, malaria,

<sup>17</sup> Mallory, F. B., *Jour. Med. Research*, 1901, vi, 264. Opie, E. L., *idem*, 1904, xii, 147.

diphtheria, and many other infections. Study of pathological tissues and experimental studies with chloroform and other substances indicate that necrosis of hepatic cells has an important part in the pathogenesis of cirrhosis of the liver. Our experiments performed with four substances which produce localized necrosis indicate that diet may exert an important influence upon the incidence and progress of similar lesions. Increased susceptibility caused by a protein diet and in some instances by fat may have a part in the production of conditions such as toxemia of pregnancy or eclampsia. The experiments suggest that carbohydrate diet may prevent their occurrence or retard their progress.

#### CONCLUSIONS.

Diet exerts a profound influence upon the toxicity (tested in the omnivorous white rat) of certain substances, namely, chloroform, phosphorus, potassium chromate, and uranium nitrate, which cause necrosis of the parenchymatous cells of the liver or of the kidney. Susceptibility to intoxication with all of these substances is less after a diet rich in carbohydrate than after a diet consisting of meat.

Carbohydrates protect the parenchymatous cells of the liver or of the kidneys from necrosis caused by any one of these substances.

Chloroform is much more toxic to animals which have received a diet consisting in great part of fat than to those which have received meat. When fat is fed to the white rat fatty infiltration occurs in the centers of the hepatic lobules and in the loops of Henle of the kidney. The necrosis caused by chloroform has the same location. The solubility of chloroform in fat determines the increased susceptibility of animals which have received fat and stored it in the parenchymatous cells of the liver and kidney.

Susceptibility to intoxication with phosphorus which causes fatty degeneration and necrosis of the liver is not increased by a diet of fat. Necrosis caused by phosphorus occurs in the periphery of the hepatic lobule and exhibits maximum intensity in animals which have received meat.

Susceptibility to intoxication with potassium chromate which causes nephritis with necrosis of the convoluted tubules of the kidney is not greater after a diet of fat than after a diet of meat.



Susceptibility to intoxication with uranium nitrate which causes nephritis with advanced necrosis of renal tubules is increased by a diet of fat. The loops of Henle, in which fat is abundant after a diet of fat, are the chief site of necrosis.

# A NEW AND RAPID METHOD FOR THE ISOLATION AND CULTIVATION OF TUBERCLE BACILLI DIRECTLY FROM THE SPUTUM AND FECES.\*

By S. A. PETROFF.

(*From the Adirondack Cottage Sanitarium, Trudeau.*)

The object of this investigation was to devise a simple, practical, and reliable method for the isolation and cultivation of the tubercle bacillus from the sputum and feces. Most of the methods employed during the last twenty years do not give uniformly positive results. In the last few years the antiformin method has proved to be of value, but it has failed to give a rapid and uniform growth. Its disadvantage rests in the fact that repeated washing of the sputum with sterile water diminishes the number of organisms and increases the possibility of contamination. Passing a specimen through an animal may or may not modify the organism. All these difficulties made it impossible up to the present to make systematic studies of tubercle bacilli bearing on cultural characteristics, staining reactions, virulence, and other biological phenomena. The work of Churchman, Krumwiede, and Simons, on the effect of various dyes upon the growth of microorganisms, suggested the use of these dyes in the isolation of the tubercle bacillus.

At first an attempt was made to grow tubercle bacilli directly from the sputum on a medium containing gentian violet. Only a few Gram-positive streptococci and diplococci appeared in the tubes. These organisms were isolated and studied. A dilution of gentian violet 1 to 1,000 failed to inhibit their growth. For the elimination of some of them sodium hydrate proved to be of greater value than antiformin.

To determine the bactericidal action of sodium hydrate on the tubercle bacillus, a series of tubes containing 1, 2, 3, and 4 per cent.

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sodium hydrate were inoculated with tubercle bacilli and left in the incubator for twenty-four hours at 37° C. At the end of that time the tubes were neutralized to litmus with normal hydrochloric acid, centrifugalized, and the sediment containing the tubercle bacilli was inoculated into tubes. After incubation for some time they showed positive growths of tubercle bacilli.

The next step was to determine what stain and dilution were most favorable for the growth of tubercle bacilli. For this purpose a series of media containing gentian violet, methyl violet, methylene blue, crystal violet, and fuchsin in dilutions varying from 1 to 1,000 to 1 to 100,000 were used. Tubercle bacilli will grow well on egg-meat juice media containing all of the above stains even in a dilution as low as 1 to 5,000, with the exception of methylene blue which gave negative results below a dilution of 1 to 25,000. In media containing this stain the tubercle bacilli grow rather slowly, and the individual organisms are impregnated with the stain. From these results gentian violet was selected as the most favorable stain, on account of its inhibitory action on many organisms.

#### PREPARATION OF THE MEDIA.

After many attempts it was found that a medium containing whole egg, beef or, preferably, veal juice, and gentian violet gave uniformly positive results. This medium contains:

Two parts of egg (white and yolk).

One part of meat juice.

Gentian violet sufficient to the proportion of 1 to 10,000.

*Meat Juice.*—500 gm. of beef or veal are infused in 500 c.c. of a 15 per cent solution of glycerin in water. Twenty-four hours later the meat is squeezed in a sterile meat press and collected in a sterile beaker.

*Eggs.*—Sterilize the shells of the eggs by immersion for ten minutes in 70 per cent. alcohol or by pouring hot water upon them. Break the eggs into a sterile beaker and after mixing the eggs well, filter through sterile gauze. Add one part by volume of meat juice.

*Gentian Violet.*—Add sufficient 1 per cent. alcoholic gentian violet to make a dilution of 1 to 10,000.

Tube about three cubic centimeters in each sterile test-tube and inspissate for three successive days: on the first day at 85° C., until all the medium is solidified, changing the places of the tubes if necessary; on the second and third days for not more than one hour at 75° C. For the bovine type omit the glycerin and infuse the meat for twenty-four hours in water. Bovine tubercle bacilli grow

in this medium even if it contains glycerin, but on account of the popular belief and the lack of data we used a medium without the glycerin.

From a careful calculation it appears that if a single organism divides in two, it will take approximately from six to seven days to grow to a pin-point colony and be visible. To confirm this, five organisms were isolated by Barber's method and inoculated in a test-tube containing gentian violet-egg-meat juice media. Every twenty-four hours the tubes were examined. On the sixth day three pin-point colonies were visible. The strain of the tubercle bacilli used in this experiment was well adapted for growth outside the body, having been isolated two years previously. This experiment shows that under most favorable conditions it will take at least six days for a single tubercle bacillus to grow to a visible colony.

#### METHOD OF ISOLATING TUBERCLE BACILLI FROM THE SPUTUM.

Fresh sputum is advisable. Equal parts of sputum (about five cubic centimeters) and 3 per cent. sodium hydrate are well shaken and left in the incubator for twenty to thirty minutes until the sputum is fairly well digested. The sputum is then neutralized to sterile litmus paper with normal hydrochloric acid, centrifugalized, and the sediment inoculated into several test-tubes containing the media described above. Neutralization is not necessary, but it is advisable. In a few instances we added to the mixture of sputum and sodium hydrate a few drops of 5 per cent. litmus solution, but this method did not give satisfactory results.

To determine the value of this method and the medium, a parallel series was carried out with a medium which did not contain gentian violet. Fifteen out of twenty specimens of the plain medium were contaminated, while all twenty gentian violet-egg-meat juice medium specimens were free from contamination. This proved that if some of the organisms were not destroyed by the sodium hydrate their growth would be inhibited by the gentian violet. Smears were made from the sediment of each specimen with the object of comparing the original with that of the growth.

The appearance of the growths was not uniform. Some specimens gave positive growth in seven days, while others took from twelve to fourteen days, but never longer. All gave positive micro-

scopical findings upon the ninth day. There was a great variation also in cultural characteristics, some being small pin-point colonies and others large flat ones. Some of the types decolorized the medium while others picked up the dye and the colonies appeared violet. Morphologically they showed considerable variation, varying from cocci to long rods. Up to the present time we have isolated tubercle bacilli uniformly from sixty-nine consecutive specimens. The sediment from six specimens, after a careful microscopic examination, was negative for tubercle bacilli; the cultures, however, were positive. The value of this method for negative sputa is being studied.

#### METHOD OF ISOLATING TUBERCLE BACILLI FROM THE FECES.

To isolate tubercle bacilli directly from the feces is not easy. There are present many spore-forming bacteria which resist the action of the sodium hydrate. The concentration of the sodium hydrate seems not so important, because a careful study showed that the length of exposure is more important than concentration. Feces are collected in wide mouthed jars; a morning specimen gives the best results. The feces are diluted with about three volumes of water and mixed well, then filtered through several thicknesses of gauze to remove solid food particles. The filtrate is saturated with sodium chloride and left undisturbed for one half hour. At the end of that time all bacteria will be found floating. This floating film is then collected with a deflagration spoon in a wide mouthed bottle, and an equal volume of normal sodium hydrate is added. This is shaken well and left for digestion in the incubator at 37° C. for three hours, in the meantime being shaken every half hour, neutralized to sterile litmus paper with normal hydrochloric acid, centrifugalized, and the sediment inoculated into several test-tubes.

The growth from the feces appears much more slowly than that from the sputum. It takes on an average from two to three weeks for the growth to appear. In all probability many of the tubercle bacilli are dead or weakened when passed with the feces.

Of thirty-two specimens studied, nineteen were positive, six were contaminated, and seven were negative. Two of the positive cul-

tures were inoculated into guinea pigs, both of which developed tuberculosis.

#### SUMMARY.

Sixty-nine positive cultures were obtained from sixty-nine specimens of sputum from practically all stages of tuberculosis. Six of these specimens were negative by direct microscopic examination, but the cultures gave positive findings. These six specimens have been positive for tubercle bacilli at some time.

Nineteen positive cultures were isolated from thirty-two specimens of feces. All these thirty-two specimens, upon direct microscopical examination, gave positive findings, some showing only a few tubercle bacilli. Six specimens were not free from contaminating organisms, and the remaining seven were negative.

The method presented in this paper has proved very simple and accurate for the isolation of tubercle bacilli from sputum. The partial success in isolating tubercle bacilli from feces may be due to the fact that many of the bacilli may be dead.

I wish to thank Drs. Lawrason Brown and F. H. Heise for their interest and helpful suggestions.

# THE FUNCTIONAL EFFECT OF EXPERIMENTAL IN- TRASPINAL INJECTIONS OF SERA WITH AND WITHOUT PRESERVATIVES.\*<sup>1</sup>

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## PLATES I TO II.

Although a rigid test during the last six years of the antimeningitis serum prepared by The Rockefeller Institute has demonstrated its therapeutic efficiency,<sup>2</sup> yet the question may be raised whether a fraction of the deaths which still occur during the process of serum treatment could not be attributed to the serum itself. At present this question must be considered of subsidiary interest, since the antimeningitis serum is the only effective remedy which we possess for this disease. However, it is obvious that every remedial agent should be perfected as far as possible, that every conceivable source of danger to the patient from the remedy itself should be carefully sought out and, if possible, removed. A few investigations of this character regarding antimeningitis serum have already been made and the present paper is an attempt in the same direction.

The main sources of danger to the organism during the repeated intraspinal administration of antimeningitis serum have been pointed out by Flexner<sup>3</sup> and are theoretically the following: anaphylaxis; the liberation of toxic products by the rapid lysis of meningococci; increased pressure in the cerebrospinal system; and intoxication from the phenol compounds often used to preserve the sera from accidental contamination. The last supposition is the one which has received the most attention, at least in this country. This focus upon the possibility that a poisoning by phenol compounds explained a number of fatalities during serum administration in meningitis is due to Dr. S. P. Kramer, who published a series of cases where death occurred after the administration of tricresol antimeningitis serum.<sup>4</sup> Kramer attributes the fatal issue to a paralysis

\* Received for publication, October 19, 1914.

<sup>1</sup> A preliminary communication has been published (Auer, J., *Jour. Am. Med. Assn.*, 1914, lxii, 1799).

<sup>2</sup> Flexner, S., *Jour. Exper. Med.*, 1913, xvii, 553.

<sup>3</sup> Flexner, *Jour. Am. Med. Assn.*, 1913, lx, 1937.

<sup>4</sup> Kramer, S. P., *Jour. Am. Med. Assn.*, 1913, lx, 1348; *Lancet-Clinic*, 1913, cix, 304, 357; 1914, cxi, 509.

of the respiration caused by the tricrosol preservative which reached the medullary centers through an opening in the conus medullaris connecting the spinal subdural space with the central canal of the cord. To support this hypothesis Kramer made a few experiments on dogs; he injected tricrosol solutions into the vertebral artery, the subdural space of the posterior cranial fossa, and into the subdural space of the lumbar region. His experiments with intravenous injections need not be considered here, because we are not dealing with the toxicity of tricrosol when given in this way. The other two experiments, illustrated each by a blood pressure and respiration tracing, showed that 2 c.c. of a 0.5 per cent. water solution of tricrosol injected in the posterior cranial fossa caused a stoppage of the respiration and a fall in blood pressure. Kramer states that artificial respiration was necessary for 10 minutes before spontaneous respiration began. In the next experiment 2 c.c. of antimenigitis serum containing tricrosol were injected into the lumbar subdural space of a young dog. A few seconds after the injection the respiration ceased but was restored partially by two short periods of artificial respiration. After this, artificial respiration was stopped and the animal died 5 minutes later.

The clinical experiences and experiments led Kramer to the conclusion that 0.5 per cent. tricrosol serum cannot safely be injected into the subarachnoid space. Kramer<sup>5</sup> suggests that tricrosol be replaced by a formalin-serum mixture of 1:1,000, and supports this by an experiment which showed that 2 c.c. of 1:1,000 formalin-serum injected into a vertebral artery of a dog produced but slight effects on the respiration and blood pressure. It must be added that, according to Kramer,<sup>6</sup> the vertebral artery delivers its contents directly to the hind-brain without passing it through the circle of Willis.

The next paper dealing with this subject is by Flexner,<sup>7</sup> who gives a critical consideration of the dangers which are referable to the serum itself. He points out that accidents have occurred after the employment of antimenigitis serum which contains no preservative at all; that injection of tricrosol serum into the lateral ventricles of the brain has caused no fatalities, though the route which the tricrosol serum could take to the medulla is surely a direct one; Flexner suggests, since the doses employed in the cases referred to by Kramer were generally maximal and were usually injected with a syringe, that increased cerebral pressure was the cause of respiratory failure in these cases.

The next investigation is by Hale<sup>8</sup> and is an experimental study of the effect of tricrosol serum upon the blood pressure and respiration, when injected intraspinally in dogs and cats. The dogs were anesthetized by 5 mg. of morphin per kilo of body-weight given subcutaneously, and by 45 mg. of ethyl carbamate and 18 mg. of chloral hydrate per kilo given *per os*. The cats received no morphin, but only the solutions of ethyl carbamate and chloral. Occasionally the anesthesia was aided by ether. It must be remarked that the hypnotics used by Hale are medullary depressants, and, in addition, at least two of them, urethane and chloral, increase markedly the secretion of cerebrospinal liquid

<sup>5</sup> Kramer, *Lancet-Clinic*, 1913, cix, 357.

<sup>6</sup> Kramer, *Jour. Exper. Med.*, 1912, xv, 348.

<sup>7</sup> Flexner, *Jour. Am. Med. Assn.*, 1913, lx, 1937.

<sup>8</sup> Hale, W., *Bull. Hyg. Lab., U. S. P. H. and M.-H. S.*, 1913, No. 91, 5.



according to Dixon and Halliburton.<sup>9</sup> Hence these two substances tend to raise the intraspinal pressure. The blood pressure was recorded from the carotid artery by a mercury manometer; the respiration was registered in a variety of ways, the final method being a cannula in the pleural cavity, connected with a tambour. For the main experiments laminectomy was performed because the results obtained by inserting the needle through the skin into the spinal dural sac were irregular.

The tricrosol serum solutions were prepared by adding the requisite amount of tricrosol (0.3 to 1 per cent.) to the antimeningitis serum about 1 hour before the injection. Just before use the mixture was filtered and warmed and injected by syringe.

The results obtained by Hale were briefly as follows: Following the injection of tricrosol serum the respiration after a preliminary stimulation slowed markedly, decreased in depth, and stopped temporarily.<sup>10</sup> After a fatal amount death was preceded by a permanent paralysis of respiratory activity, though certain experiments showed respiratory gasps as long as the heart continued to beat.<sup>11</sup> The blood pressure often showed a slight initial rise, but in every instance it later fell below normal, while the heart rate was not much changed. After a toxic amount the blood pressure fell slowly to zero subsequent to the failure of the respiration.<sup>12</sup>

The amount of tricrosol serum which caused death Hale found to be less than 2 c.c. of a 0.5 per cent. tricrosol solution per kilo of body-weight. With 0.3 per cent. solutions the fatal dose was "somewhat larger"; with 1 per cent. "considerably smaller."<sup>13</sup>

Hale states<sup>14</sup> that normal serum never produced symptoms aside from those ascribed to increased cerebral pressure: the blood pressure always shows an enormous rise which later rapidly falls to zero in the fatal cases; associated with this is a complete cessation of respiration. After tricrosol serum the respiration fails, but the "blood pressure always falls" and does not show the great rise obtained with serum alone. Moreover, serum containing tricrosol was much more toxic than the same serum free from preservative.

Hale concludes<sup>15</sup> that his experiments substantiate Kramer's contention that tricrosol is a dangerous preservative for sera which are to be brought directly into contact with the vital nervous centers, and that death may result either from an increase of intracranial tension or from the presence of tricrosol in the serum. The danger from tricrosol, however, according to Hale, "seems much greater and more certain than that which may possibly arise from increased pressure."

The last paper on this subject is a short communication by Fitzpatrick, Atkinson, and Zingher.<sup>16</sup> These authors made a series of experiments on dogs

<sup>9</sup> Dixon, W. E., and Halliburton, W. D., *Jour. Physiol.*, 1913-14, xlvii, 238.

<sup>10</sup> Hale, *loc. cit.*, p. 10.

<sup>11</sup> Hale, *loc. cit.*, p. 14.

<sup>12</sup> Hale, *loc. cit.*, p. 14.

<sup>13</sup> Hale, *loc. cit.*, p. 14.

<sup>14</sup> Hale, *loc. cit.*, p. 17.

<sup>15</sup> Hale, *loc. cit.*, p. 18.

<sup>16</sup> Fitzpatrick, C. B., Atkinson, J. P., and Zingher, A., *Proc. Soc. Exper. Biol. and Med.*, 1914, xi, 182.

by injecting tricresol and chloroform sera intravenously, intra-arterially, and into the lumbar subdural space. Only the latter experiments need be considered here. The injections were made through the skin, and laminectomy was not performed. They observed no marked disturbances of the blood pressure in normal dogs after moderate doses of antimeningococcus sera containing 0.3 per cent. tricresol. Similar results were obtained when physiological salt solution, "old" antimeningococcus serum, antimeningococcus serum with 0.3 per cent. and 0.4 per cent. tricresol, normal horse serum, and various sera with chloroform were injected subdurally. They conclude that pressure appears to be a factor of real danger.

The investigation to be reported in the following pages was undertaken in order to study not only in dogs, but also in monkeys, the effects which are produced when normal sera or sera preserved by 0.3 per cent. tricresol, 0.3 per cent. chloroform, and 0.3 per cent. ether are injected into the lumbar subdural sac. The tricresol and chloroform sera were of this strength because the majority of the dog experiments and all the monkey experiments were carried out with the antimeningitis sera furnished by the New York and Massachusetts Boards of Health, and in these sera the preservatives are present in 0.3 per cent. strength.

Because of the opinions already expressed by various authors concerning the action of sera when injected intraspinally, the experiments made in this investigation will be given with some detail and they will be illustrated by curves which show the behavior of an animal during a fifteen minute interval.

#### EXPERIMENTS.

*Method.*—All experiments were carried out in dogs and monkeys (*Macacus rhesus*). Both species of animals were kept under light ether anesthesia and the loss of body heat was prevented or reduced by an electric pad upon which the animal was stretched out. The method of maintaining anesthesia as well as the preliminary operative interferences were, however, quite different in the dog and the monkey, although it was attempted to make the conditions of the experiments as similar as possible to those which prevail when the human subject receives an intraspinal injection of a therapeutic serum. It will be seen from the following descriptions that only in the monkey were the anatomical conditions such as to permit a close approximation to the procedure employed in human therapeutics.

The dog was always anesthetized by means of intratracheal insufflation, a procedure which not only permits the maintenance of a quite constant level of narcosis, but which also prevents any danger from the stoppage of spontaneous respiration. In the monkey no intratracheal insufflation was attempted

and the animal was etherized cautiously by the ordinary cone-drop method. The latter animal, therefore, received a varying amount of ether per unit of time due to the method, and was subject to asphyxia if the respiration should become shallow or fail. Tracheotomy was only performed in one series of dogs where local applications were made to the medulla.

Another wide divergence of procedure occurred in the process of injecting the alien serum subdurally. In the dog the ordinary method employed in the human being cannot be used with safety and certainty because the spinal cord of the dog, unlike that of the monkey, extends practically into the sacrum. If a hypodermic needle is introduced between the lumbar vertebral laminæ, it is thus almost certain to wound the spinal cord, and the damage would be increased if followed by an injection; if the insertion of the needle is made in the neighborhood of the sacrum there is little guarantee that the needle is really in the dural sac, for spinal fluid was obtained only exceptionally no matter where the needle was introduced. These difficulties render an exposure of the spinal cord imperative even for moderately precise work of this type in the dog, and a laminectomy was therefore always performed in this animal. Hale also found a laminectomy necessary in the dog in order to secure uniform results.<sup>17</sup>

Under ether anesthesia two lumbar vertebræ were removed, either the second and third or the third and fourth. After exposure of the dura the wound was carefully covered with a fine layer of dry cotton to prevent oozing, and the cavity was filled with a wad of cotton saturated with vaselin. The injection was made by means of a hypodermic needle bent at right angles. After preliminary puncture of the dural sac the needle was introduced and the dura gently lifted so that the injected fluid should not injure the cord. After injection the needle was usually kept *in situ* for some seconds, then withdrawn, and the puncture wound immediately covered firmly with the vaselin-soaked cotton plug, and this plug itself was kept applied by means of a light weight.

In the monkey, however, no such severe measures were necessary in order to give an intraspinal injection with certainty; here it sufficed to introduce a hypodermic needle between the laminæ of the lumbar vertebræ in order to tap the spinal dural sac. The appearance of spinal fluid in the cap of the hypodermic needle served as an index of the proper location of the needle point. Under no circumstances was an injection ever given to a monkey before clear or only slightly blood-stained spinal fluid had been obtained. Occasionally, however, the amount of spinal fluid available apparently was too small to fill the hypodermic needle, which had a lumen of about 0.5 mm. Such a diminution of available spinal fluid seemed to occur especially in monkeys which had been injected some days previously, and was only exceptionally encountered in unused monkeys. Under these conditions a sterile capillary glass tube was cautiously inserted into the needle; if fluid was present it rose at once in the tube and even formed a drop at the free end. It need hardly be stated that for this test the hypodermic needle must be inserted dry.

The site of injection in the monkey varied; usually the space just above the one at the level of the iliac crests was employed; if no fluid was obtained, the space above or below the one mentioned was entered. Occasionally it

<sup>17</sup> Hale, *loc. cit.*, p. 11.

happened that the first three attempts failed, but later trials in a space already tested yielded cerebrospinal fluid, at times in abundance.

As the majority of the monkeys were reinjected after 7 to 15 minutes when graphic records of the blood pressure and respiration were taken, the hypodermic needle tapping the spinal dural sac was allowed to remain *in situ* in the etherized monkey throughout the experiment. After cerebrospinal fluid had been undoubtedly obtained, the cone-shaped orifice of the cap was stoppered with a cork. Repeated intraspinal injections were thus rendered a simple matter, and only a slight loss of fluid took place before the stopper could be replaced.

The injections were always given by syringe and never by the gravity method, in order to test the sera under conditions which obtain when an intraspinal injection is given by the average physician. It may be remarked that there seems to be a general impression that the gravity method abolishes the danger of increased intraspinal pressure. This is not true unless the serum is absorbed as soon as injected, a condition which is probably never fulfilled. The pressure under which serum enters the dural sac must therefore always be greater in the gravity system than in the spinal dural sac, for otherwise no serum would enter; in consequence the intraspinal pressure must also rise when the rate of infusion exceeds its rate of absorption, and the degree of this rise of intraspinal pressure depends entirely upon the operator.

The blood pressure was always recorded by a mercury manometer, the tubing being filled with a half saturated solution of sodium sulphate. In the dog the left carotid artery was used, and in the monkey a femoral artery.

The respiration was written in both dogs and monkeys by means of a Meltzer pleural cannula which was inserted in the left pleural cavity and connected with a Marey tambour. The negative pressure was often reestablished after the pleural cannula was fixed in place by distending the lung and then closing the cock of the cannula. Occasionally it happened that the cannula was improperly placed so that its orifice became plugged; when this occurred a partial or even full unilateral pneumothorax was established in order to obtain a graphic record.

The following kinds of horse serum were injected: 0.3 per cent. tricesol antimeningitis serum (no other strength was tested), 0.3 per cent. chloroform antimeningitis serum, and 0.3 per cent. ether horse serum. For the tricesol and chloroform antimeningitis sera I am under obligation to the New York and Massachusetts Boards of Health, which placed liberal quantities of these sera at my disposal. The ether serum was prepared by the addition of an appropriate amount of ether to the cold sterile horse serum in a tightly stoppered bottle which was then shaken and kept in the ice-chest. Sterile horse serum without any preservative whatever or Ringer solution was also injected as controls. Practically none of the sera employed were freshly prepared. All were usually at least several weeks old before they were used for intraspinal injection. A few experiments, however, in the dog were made with normal horse serum to which 0.3 per cent. tricesol had been added, and this serum was used after a few days. In these experiments more severe effects on the blood pressure and respiration were obtained than with 0.3 per cent. tricesol antimeningitis

serum. As only a few experiments were made with this serum, it can merely be suggested that fresh tricresol sera perhaps exert stronger effects upon the medullary centers than older solutions.

The usual amount injected each time was 5 c.c. in the dog and 3 c.c. in the monkey. In the dog a certain leakage always occurred after the second injection through the puncture made in the dural sac by the hypodermic needle. This leakage was reduced by immediately covering the puncture and exposed dura with a vaselin-cotton plug. In the monkey a slight subcutaneous leakage from the dural sac was observed only once.

The dose injected per kilo of animal ranged from 2 to 6 c.c. for both dogs and monkeys. It must be observed that this method of calculating the amount of serum injected intraspinally is not a good one, for the size of the dural sac by no means increases in the same ratio as the weight of the dog. If thus two grown dogs of 5 and 10 kilos' weight received each 25 c.c. of serum intraspinally, the statement that one received 5 c.c. per kilo and the other 2.5 c.c. per kilo is likely to cause a wrong impression, unless the weight of the dog is noted.

In order to study the effects of various substances when applied directly to the medulla without pressure, a number of dogs were prepared by exposing the medulla, the blood pressure and respiration being recorded as usual. The animals were placed on the abdomen with the head sharply flexed ventrally, and the medulla was exposed in this position by carefully cutting a window in the occipito-atlantoid membrane. Removal of this window allows, as a rule, a small amount of cerebrospinal liquid to escape, and the floor of the fourth ventricle is then easily seen. Occasionally the excision of the membrane caused a hemorrhage by wounding a vein; when this occurred the animal was rejected because the red corpuscles gradually settled and formed a layer over the surface of the fourth ventricle which delayed absorption. The animals were kept under ether by means of intratracheal insufflation, but the tracheal catheter was inserted through a tracheotomy wound in the neck so as to avoid an overdistension of the lungs which could be caused by a compression of the trachea due to the extreme ante flexion of the head. No experiments of this type were made on the monkey.

No aseptic precautions were taken when operating on the dog, for they were always killed painlessly before removal from the operating table by allowing a quantity of ether to enter the trachea. In the monkey, however, full strength tincture of iodine was used to disinfect the skin before operating. After completion of the experiment the wounds were lightly wiped with the tincture and sutured with cotton ligatures which had been soaked in tincture of iodine. No bandages of any kind were used, and the wounds were never sealed with collodion. The results were excellent and suppuration was never seen. Most of the monkeys were used at least twice.

In one series of three monkeys the effect of intraspinal injections was studied by inspection only. The animals were held by hand and stretched over the knee of an assistant; the hair over the lumbar spinal region was clipped, the area painted with tincture of iodine, and then the intraspinal injection was given after first obtaining cerebrospinal liquid. This method of injection is unsatisfactory because it interferes considerably with respiration, especially if the animal struggles.

From the preceding description of the procedures employed to study the effects obtained when various liquids are injected into the spinal canal, it will readily be seen that the conditions obtaining in the monkey experiments approximate much more closely the conditions under which intraspinal injections are given in the human being, than do the experiments made on dogs. The latter animal unfortunately demands a severe operation on the spinal column before injections can be given with any certainty. The results obtained from the monkey are, therefore, *a priori* entitled to more weight than those obtained in the dog, and this inference is strengthened when it is observed that both man and the monkey seem to react in general quite similarly to intraspinal injections of sera.

#### RESULTS.

In general it may be said that intraspinal injections of sera with and without preservatives (0.3 per cent. tricresol, 0.3 per cent. chloroform, 0.3 per cent. ether) produce qualitatively the same effect upon the medullary centers in the monkey and the dog. The effects differ, however, quantitatively with the preservative employed, with the dosage, and with the species of animal used, the monkey being strikingly more resistant than the dog. Broadly stated, the respiration is first increased both in amplitude and rate for a short period of time; then, particularly in the dog, the amplitude decreases and the rate slows and the respiration may even stop for a shorter or longer period of time. The blood pressure shows an initial rise followed by a gradual drop of varying degree. This drop is usually associated with a marked slowing of the pulse rate in the dog. The normal level is again reached after some minutes.

In the following pages a fairly detailed description of the functional disturbances caused by the intraspinal injection of the liquids mentioned will be given, illustrated by specimen curves of the respiration and blood pressure, especially those which have been obtained from the monkey. In order that these tracings may be as complete and comprehensive as possible, most of them will show a fifteen minute portion of an experiment.

#### EXPERIMENTS ON MONKEYS.

*Tricresol Series.*—0.3 per cent. tricresol antimeningitis serum obtained from the New York Board of Health was used. Eight monkeys were used; in four

the intrapleural pressure (respiration) and blood pressure were recorded; three of these four were used twice on successive days. The remaining three monkeys were held by hand, injected intraspinally with serum, and then liberated and observed. These injections were repeated a number of times on the same day and on succeeding days. The monkeys, as a rule, received 3 c.c. of the serum per injection, but occasionally 5 c.c. were given.

The first three monkeys, which varied in weight from 2,065 to 2,560 gm., were studied by inspection only, the animals being held across the knee of an assistant during an injection. No ether was administered. After each injection they were released but kept under control by means of a cord attached to the animal's belt. The injections of serum were given intraspinally at rather long intervals, varying between 30 and 90 minutes, in general. The animals were also reinjected on succeeding days. It developed that the injections usually produced only a moderate temporary disturbance characterized chiefly by a muscular weakness, so that the animal usually was content to remain quietly in one place; associated with this was an initial slowing and deepening of the respiration followed by a more rapid and shallower respiration. None of the animals died, but all were in excellent condition more than one month after the last injection. The amounts of serum injected in one day were as follows:

Monkey 1 was a male, weighing 2,560 gm. On Mar. 30 it received three times 2 c.c. and once 4 c.c. of tricrosol serum. The next day it received one injection of 3 c.c. The next day (Apr. 1) two doses of 3 c.c. each were injected. Two days later three injections of 3 c.c. each were given, and 2 days after this two doses of 3 c.c. each. The total amount injected was 34 c.c. in twelve doses.

Monkey 2 was a female, weighing 2,400 gm. On Mar. 31 it received two intraspinal injections of 3 c.c. each; the next day one dose of 3 c.c.; 2 days later (Apr. 3) two doses of 3 c.c. each; and three days later (Apr. 6) two final injections of 3 c.c. each. The total amount was 21 c.c. given in seven injections.

Monkey 3, a male, weighing 2,065 gm., received on Apr. 4 two intraspinal injections of serum, of 3 c.c. each. Two days later the same dose was repeated for the last time. This animal received only 12 c.c. in four injections.

During these twenty-three injections of serum a collapse was observed a number of times under two apparently different conditions: (a) after recovery from preceding injections, the repeated insertion of the hypodermic needle, without injection of serum, caused a collapse of varying degree four times; and (b) after the repeated injection of tricrosol serum a moderate collapse occurred twice. These effects were observed only in monkeys 1 and 2.

The third monkey exhibited no definite collapse at any time while its spinal dural sac was tapped or after receiving the injection of tricrosol serum.

It is impossible to state what part, if any, the tricrosol serum played in producing this sensitiveness to the introduction of a needle into the spinal subdural space, for no other experiments of this type with other sera were carried out. It is possible that asphyxia was a contributory factor, for the monkeys which showed a collapse always struggled vigorously when the needle was introduced; in consequence they had to be held firmly pressed over the knee of the assistant, and this interferes considerably with respiration.

In the next series of five monkeys (4, 5, 6, 7, 8) the 0.3 per cent. tricrosol serum was injected more rapidly and regularly than in the preceding series, and

the animals were all etherized by the cone method. The interval between injections was either 7 or 15 minutes; in the later experiments it was usually 7 minutes. No intratracheal insufflation or other artificial respiration was used as a routine measure in any of the monkey experiments. In all these experiments except monkey 8 the blood pressure and respiration were recorded on a smoked drum. The quantities of serum injected were large. Monkey 4, a female, weighing 3,455 gm., received 18 c.c. the first day and 28 c.c. the next day; it was killed later in excellent condition. Monkey 6, a male, weighing 2,926 gm., received 15 c.c., and the next day tolerated without difficulty 18 c.c. of serum and 5 c.c. of Ringer solution. This animal was also killed later in excellent condition. Monkey 7, a female, weighing 2,865 gm., received intraspinally 24 c.c. of tricrosol serum without any alarming effects. The next day it was in good condition, but died four days later from dysentery which it had contracted before the experiment. Monkey 8, a female, weighing 2,850 gm., was fully anesthetized with ether and then injected with tricrosol serum without recording its blood pressure or respiration. This animal received intraspinally four doses of serum of 3 c.c. each at 10 minute intervals without any marked effect on the respiration beyond the initial deepening and slowing of the respiration usually observed during an injection. Thirty minutes after being placed in a cage it was lively. The next day the same animal was again etherized and received 12 c.c. of 0.3 per cent. chloroform antimenigitis serum intraspinally in 3 c.c. doses with no marked effects. Nine days later the same animal again received 12 c.c. of 0.3 per cent. chloroform serum while under ether without any evil effects. Three weeks later the same animal seemed perfectly normal.

Monkey 5, a female, weighing 3,225 gm., succumbed after receiving only 9 c.c. of tricrosol serum. This was the only death which occurred in the tricrosol monkey series and its causes will be considered later (p. 54).

The effect which 0.3 per cent. tricrosol antimenigitis serum exerts upon the blood pressure and respiration of monkeys can best be shown by illustrative tracings (figures 1, 2, 3). These three sets of curves show each a period of about 15 minutes, and were all obtained from the same animal, monkey 4. Figure 1 shows the effect of the first injection upon the blood pressure and respiration: the former shows a strong initial temporary rise during the intraspinal injection which is promptly followed by a drop to the 70 mm. level within 4 minutes after the injection. The normal level of about 100 was reattained about 3 minutes later. The heart beats were moderately slowed during the stage of lowered pressure. The respiration was first increased both in rate and amplitude, and then the rate slowed moderately. Figure 2 shows a tracing from the same animal after it had already received 15 c.c. of 0.3 per cent. tricrosol serum. The next 3 c.c. of the serum, 18 c.c. in all, now produced but slight effects on the blood pressure and respiration: the blood pressure fell 17 mm. after the injection and recovered its original level swiftly. The heart beat was again moderately slowed. Ten minutes after this last injection, when 18 c.c. of the serum, more than 5 c.c. per kilo of body-weight, had been incorporated, the animal was perfectly normal as far as blood pressure and respiration were concerned. The thoracic cannula was then removed and the wound sutured, leaving a one-sided pneumothorax. The right femoral artery was ligated and the incision in the groin sutured. Within 1½ hours the monkey was moving



about actively in its cage. The next day the same animal, in excellent condition, was again etherized and prepared for experiment. The respiration was recorded through the same wound in the left chest as on the day before; the blood pressure was registered from the left femoral artery. Now the animal received five injections of 3 c.c. of tricrosol antimeningitis serum at 15 minute intervals with only slight effects on the blood pressure and respiration. The sixth injection of 3 c.c. caused a slow marked fall of blood pressure from 100 to 51 mm., but full recovery (98 mm.) took place within 7 minutes after the injection, although this last injection was given much more rapidly than the preceding ones. The seventh dose consisted of 5 c.c. of serum injected more rapidly than the doses of 3 c.c. The blood pressure dropped promptly from 94 to 44 mm., but again showed swift recovery within 7 to 8 minutes. The respiration showed a moderate temporary decrease in amplitude and increase in rate. Figure 3 depicts this seventh injection, the animal having tolerated so far 23 c.c. of tricrosol serum. The eighth injection also consisted of 5 c.c. given within 15 seconds; the respiration slowed moderately at first, then accelerated markedly (from the normal rate of 65 to 100) with a moderate diminution in amplitude; it never caused any apprehension. The blood pressure, however, dropped promptly after a short initial rise from 110 to 34 mm.; recovery began after about 6 minutes and was then accelerated needlessly by the intraspinal injection of 2 c.c. of adrenalin. This injection drove the blood pressure to 200 mm. within 1.5 minutes after the injection. Ten minutes later the respiration was 80 per minute, of good depth, and the blood pressure recorded 150 mm. The animal was in excellent condition and was now anesthetized to death. This animal had, therefore, received in all 28 c.c. of 0.3 per cent. tricrosol antimeningitis serum, or 23 c.c., if we exclude the last dose because the recovery was hastened by adrenalin. This latter amount represents the enormous dose of 8 c.c. of serum per kilo of body-weight, a dose which was borne with perfect safety. It must be remembered that the same animal received the day before 18 c.c. of tricrosol serum (5.2 c.c. per kilo) and had a left-sided pneumothorax when used for the second time.

This remarkable tolerance was not an isolated instance, but was also exhibited by monkey 6 of the tricrosol series, a male, weighing 2,926 gm. It was treated exactly like monkey 4. On the first day 15 c.c. of serum were injected in 3 c.c. doses at 15 minute intervals with effects similar to those observed in figure 1. At the end of the experiment when 5 c.c. of serum per kilo of body-weight had been introduced, the animal was in excellent condition. The operation wounds in the chest and right groin were wiped as usual with tincture of iodine and then sutured with cotton ligatures soaked in tincture of iodine. This animal, as a result of the method, also had a left-sided pneumothorax; nevertheless, within one hour after being placed in the cage it was active and in excellent condition.

The next day this monkey, in excellent condition, was utilized for another experiment of the same type. The pleural cannula was inserted through the same opening in the left chest which had served the day before; the blood pressure was recorded from the left femoral artery. The monkey then received intraspinally six times, at 15 minute intervals, 3 c.c. of the serum. The effect on the blood pressure was even less than on the day before the drop, not

exceeding 20 mm., and the normal level was always reattained within 3 minutes. The respiration showed the usual alterations of rate and amplitude already described. During each injection, however, the animal showed convulsive general movements and gasping respirations with stoppages in active expiration, which ceased as soon as the injection was completed. There was also a slight general tremor which lasted less than a minute after the injection. After the sixth serum injection the animal was in very good condition, and 5 c.c. of Ringer solution were injected intraspinally as rapidly as possible. A tonic convulsion set in at once associated with slow, gasping respirations. These symptoms, however, swiftly grew less and after 5 minutes the monkey was as well as before. He was then killed by ether. The animal had therefore received with safety 5 c.c. per kilo of body-weight of 0.3 per cent. tricresol antimeningitis serum intraspinally on one day and 6 c.c. of the same serum per kilo on the next day.

In monkey 7 of this series the injections of tricresol antimeningitis serum were given at 6 to 8 minute instead of 15 minute intervals, as in other animals, in order to make the test still more severe. This was a female animal, weighing 2,865 gm. It was prepared for the registration of respiration and blood pressure as the other monkeys. 3 c.c. of the serum were injected eight times intraspinally in this animal at short intervals without producing anything but a temporary disturbance of the respiration and blood pressure during and shortly after the injection. Figure 4 shows the slight effects produced by the seventh and eighth intraspinal injections of 3 c.c. each. In addition to the enormous dose of 8 c.c. per kilo of animal, which was tolerated without danger, 3 c.c. of 0.3 per cent. chloroform antimeningitis serum and 3 c.c. of sterile horse serum without any preservative were given intraspinally without causing any dangerous symptoms. The animal was in excellent condition at the end of the experiment, which had lasted about one hour and a half, the time consumed by the operative preparations not included. The wounds in the chest and groin were now sutured, and the animal was returned to its cage. Two hours later it was in good condition and the next day (May 8) it moved about and ate. On May 11, 4 days after the experiment, it looked sick and fluid feces mixed with blood were found in the cage. In the afternoon of the same day the animal died. Investigation showed that the animal had a colitis, for its gut from the cecum to the rectum showed petechial hemorrhages in the mucosa with occasional erosions; the small gut was clear. No pus or excess fluid was found in the chest; the wound in the chest wall was entirely closed by a plate of fibrin. This animal was taken from a group a number of which died from dysentery; its death, however, from dysentery may have been aided by the effect of the operation.

In monkey 5 of this series a number of technical difficulties were encountered. This animal was a female, weighing 3,225 gm., and was apparently in heat. It was so readily etherized that too much ether was given (cone method) and respiratory recovery was slow and not complete. The placement of the pleural cannula was faulty so that registration only became possible after establishing a full left-sided pneumothorax. In addition to these mishaps difficulty was encountered in obtaining spinal fluid. The hypodermic needle was inserted five times in three lumbar spaces before undoubted spinal fluid was obtained.

Although the animal was in poor condition before any injection was given, nevertheless it tolerated two injections of tricrosol serum of 3 c.c. each without any difficulty; the respiration remained fairly satisfactory and the blood pressure, after dropping from 90 to 42 mm. after the first injection, and from 94 to 52 mm. after the second dose, both times regained its normal level (90 to 95 mm.) within about 8 minutes after the injection. The third dose, however, produced during its injection a respiratory stoppage in active expiration lasting about 10 seconds, followed by series of convulsive, deep respirations with active expiration; subsequent to this the respirations were inspiratory jerks of short duration followed by pauses in passive expiration. These respiratory attempts were about 30 to the minute, while the normal respirations had numbered 70. The blood pressure had risen moderately during the injection and then dropped slowly to the 34 mm. level in 4 minutes, the respiratory jerks at that time numbering 30 per minute. The heart rate had slowed at once after the injection, and 4 minutes later was 155, while the normal rate before injection was 220 per minute. While the heart was still beating regularly, the respiration suddenly ceased entirely and the blood pressure continued to fall. Pharyngeal insufflation was begun but did not resuscitate the animal. Unfortunately no attempt was made to withdraw some of the spinal fluid in order to decrease spinal pressure. Had this been done it seems probable that the animal would have survived. Evidence for this statement will be given later (figure 8). Monkey 5 was the only one of the entire series of eight which succumbed to the intraspinal injection of tricrosol serum.

*Chloroform Series.*—Three experiments on two normal monkeys were carried out with 0.3 per cent. chloroform antimenigitis serum obtained from Dr. Theobald Smith. The chloroform serum was not warmed before injection. The monkeys were prepared as usual for recording the blood pressure and respiration.

Monkey 1 was a male, weighing 2,045 gm., and received at 7 minute intervals four doses of 3 c.c. each intraspinally. The effect on the blood pressure was slight; after a short initial rise not exceeding 15 mm. the pressure declined slightly, not more than 10 mm., and within 5 minutes the pressure was at practically the same level as before injection. The respiration slowed slightly after the injections. Then two doses of 3 c.c. each of tricrosol serum were injected at 7 minute intervals, which also did not affect the blood pressure and respiration to any extent. It must be mentioned that in this experiment the skin above the site of injection showed a slight non-hemorrhagic, boggy infiltration, as if some of the injected serum had escaped from the subdural space. This animal was killed by ether immediately after the experiment.

Monkey 2 of the chloroform series was a male, weighing 2,835 gm., and was prepared as usual for graphic registration. The animal received five injections of 3 c.c. each intraspinally at 15 minute intervals, except the last two which followed after 7 minutes. The effect on the blood pressure was negligible except perhaps after the third dose when the blood pressure slowly sank 15 mm., but recovered its normal level of 90 mm. within 5 minutes. Two injections of 3 c.c. each of tricrosol serum were given intraspinally at 7 minute intervals. These two injections hardly affected the respiration; the blood pressure sank less than 15 mm., and the normal level was reached 6 minutes later. Seven minutes after the last injection of tricrosol serum the respiration was 55, the

blood pressure 90. The wounds were now sutured and the monkey placed in its cage. Five hours afterwards the animal ate.

On the next day the same animal, in excellent condition, was again etherized and prepared for registration. The experiment lasted over 2 hours and during this time the animal received at 7 minute intervals (except the second which followed the first injection after 15 minutes) 23 c.c. of chloroform serum, 21 c.c. of tricresol serum, and 10 c.c. of Ringer solution. After this large dose the monkey was in good condition, its blood pressure was 77 mm., its respiration 65, and its heart beats numbered 180 to the minute. In order to be certain that the injections had really been subdural, 10 c.c. of a warm gelatin solution colored with methylene blue were injected through the needle tapping the subdural space (the needle had remained *in situ* throughout the experiment). After this injection the animal was killed by an excess of ether. Autopsy showed, contrary to expectation, that the dural spinal sac was not distended with fluid. After slitting the dura and exposing the brain, the sensory and motor nerve roots were found to be a deep blue; the surface of the cerebellum and cerebrum showed a lighter blue. No blue color was observed in the lateral ventricles or the fourth ventricle, though these areas were left exposed to the air for some hours. There can, therefore, be no doubt that the large quantity of liquid injected during the experiment had all entered the spinal subdural space.

The effects produced by a series of injections of chloroform serum in monkey 2 used for the second time were similar to those already recorded; there was practically no effect on the respiration, the amplitude diminished very slightly for some minutes after the injection with occasionally a slight slowing of the rate; the blood pressure showed a moderate, gradual drop which only once exceeded 20 mm. (figure 5), and the level before injection was always attained within 6 minutes after the injection. Figure 5 illustrates this clearly: these two curves were obtained after the animal had already received 15 c.c. of chloroform serum and 6 c.c. of tricresol serum. Figure 6, obtained from the same animal after it had received 18 c.c. of chloroform serum and 6 c.c. of tricresol serum, shows clearly the difference between the effects of tricresol and chloroform antimenigitis serum, although the drop in blood pressure with the chloroform serum is greater than that usually observed. The chloroform serum caused a drop of 16 mm. in the blood pressure followed by a prompt steady recovery of its former level; the tricresol serum brought on a profound drop of 38 mm. followed by a slightly slower recovery. The respiration after the chloroform serum was only slightly affected, and that in the direction of a slowing, with only a slight change in the amplitude; after the tricresol serum the respiration showed a definite temporary decrease in its amplitude with an increased rate which tended to persist for some time.

These experiments on monkey 2, weighing less than three kilos, demonstrate strikingly what large doses of serum with chloroform and tricresol as preservatives can be tolerated intraspinally without danger: on one day the monkey received 15 c.c. of chloroform serum and 6 c.c. of tricresol serum; on the next day the same animal readily tolerated 21 c.c. of chloroform serum, 23 c.c. of tricresol serum, and 10 c.c. of Ringer solution (figures 5 and 6).

*Ether Series.*—Two experiments were made on two monkeys with 0.3 per cent. ether horse serum.

Monkey 1 was a male, weighing 2,965 gm., and was prepared as usual for recording. It received 12 c.c. of ether serum in 3 c.c. doses at 7 minute intervals. The injections not only caused no fall of blood pressure, but the initial rise of blood pressure which follows all intraspinal injections in the monkey was maintained for some minutes more or less, and then the pressure reached practically the same level as before the injection. The respiration in this animal exhibited rhythmical fluctuations in amplitude, but no marked changes in rate. It must be stated that this animal received too much ether during the operative procedures, and its general condition was not satisfactory when the injections were begun. Moreover, a full pneumothorax had to be established on the left side in order to obtain records of the respiration. Nevertheless the monkey was in good condition after it had received more than 4 c.c. per kilo of ether serum at the end of the experiment. After suturing the wounds in the left pleura and groin the monkey was placed in a cage, and within one hour it moved about actively. Recovery was complete, and 24 days later it weighed 200 gm. more than on the day of experiment.

Monkey 2 of this series was a male, weighing 3,575 gm., and was prepared as usual for records. It received six injections of 3 c.c. each of ether serum at 7 minute intervals, a dose of more than 5 c.c. per kilo of body-weight. The blood pressure, as in monkey 1, showed no fall, but the same tendency to maintain for some time the initial rise of pressure caused at once by the injection. Several minutes after the injection the pressure usually reached the original level before injection and did not fall below it. No marked alterations occurred in the respiration either in rate or amplitude, except that the respiration became somewhat deeper after 4 c.c. had been injected. The curves of figure 7 show the effects described; they were obtained after the monkey had already received 12 c.c. of ether serum.

While suturing the pleural wound, leaving a full left pneumothorax, the respirations became shallow, gasping, and the face grew gray. The ether was discontinued at once and the suture of the chest hastily completed. Although the respiration improved a little, it was considered best to give pharyngeal insufflation<sup>18</sup> for a few minutes. The color of the tongue swiftly became pink, and the spontaneous respirations grew deeper and more frequent. After 5 minutes the pharyngeal insufflation was discontinued and the animal now breathed well spontaneously. It was placed in a cage and 2 hours later moved about quite actively. Eight days later it appeared in good general condition, although the skin wounds had opened. Twenty-five days after the operation it was found dead. Autopsy showed a right-sided pneumonia. There was no pus in the left chest where the pleural cannula had been inserted, but the left lung was largely atelectatic. The cord seemed normal; there were no adhesions or signs of inflammation. It is hardly necessary to state that this death cannot be attributed to the intraspinal injections.

*Sterile Horse Serum Series.*—In these experiments sterile horse serum without any preservative was employed. The serum was several months old. Two experiments were made in 2 days on one monkey. This animal was a

<sup>18</sup> Meltzer, S. J., *Jour. Am. Med. Assn.*, 1913, 1x, 1407. See also Committee Report, *Bureau of Mines, Technical Papers*, 1914, No. 77, 28.

male, weighing 3,295 gm. After the usual preparation 15 c.c. of sterile horse serum and 3 c.c. of tricresol serum were injected intraspinally in 3 c.c. doses at 15 minute intervals. The sterile horse serum caused hardly any alteration in the blood pressure or respiration, beyond the ordinary initial effect during and slightly after the injection. The injection of 3 c.c. of tricresol serum, however, caused a prompt drop of about 30 mm. of mercury, but recovery was practically complete after 10 minutes. The respiration this time was slowed by the tricresol serum from 80 to 60 per minute. Twelve minutes after this last injection the animal was in satisfactory condition: the blood pressure was 76 mm., the heart rate 180, and the respirations were 65 per minute. Suture of the wounds was completed without any mishap, and the animal was removed to its cage where it sat up at once. During the next 3 days it was in excellent condition, and on the third day it was again prepared for an experiment. Four injections of 3 c.c. each of sterile horse serum were injected intraspinally at 15 minute intervals without practically affecting the blood pressure and respiration. The fifth injection was 3 c.c. of tricresol serum given 15 minutes later. The blood pressure dropped promptly from 98 to 54 mm., the pulse rate remaining at 225 to 230, while the respiration slowed from 100 to 70 per minute, the amplitude remaining the same. After 15 minutes full recovery of the original blood pressure, pulse rate, and respiratory rate had occurred. 3 c.c. of sterile horse serum without any preservative were injected with the usual absence of any effect on the blood pressure and respiration. Twelve minutes after this injection another dose of 3 c.c. of tricresol serum were injected intraspinally: the blood pressure rose slightly (10 mm.) and then dropped within 2 minutes to the 20 mm. level; the respiration after the initial quickening slowed to 20 per minute (original rate 95) and was feeble; occasionally deeper gasps occurred. As this condition did not improve, pharyngeal insufflation was begun 2 minutes after the injection, and a short time later the spinal fluid was allowed to escape by removing the cork in the needle cap. The blood pressure gradually improved and the respiration became stronger. Ten minutes after the beginning of pharyngeal insufflation it was discontinued; spontaneous respiration was now of excellent depth and numbered 40 to the minute; the blood pressure was 46 mm. at this time; 7 minutes later it reached 63 mm., and the respirations were 55 per minute. Now 3 c.c. of sterile horse serum without any preservative were injected intraspinally; the blood pressure rose to 69 mm. and remained at that level; the respiration was not altered in rate, but the amplitude became very slightly less. Five minutes later 3 c.c. of 0.3 per cent. tricresol serum were injected with the same result as the other tricresol serum injection: the blood pressure showed the usual initial rise and then dropped slowly within the next 3 minutes to the 18 mm. level; the pulse rate sank from 230 to 185 and then to 90 (cardiac block), the respiration after a short quickening slowed from 55 per minute to 4 per minute, and was gasping in character. The intraspinal pressure was reduced by removing the cork in the hypodermic needle and clear fluid began to drip away rapidly. No artificial respiration of any kind was given. In about 30 seconds the blood pressure began to rise, the respirations became quicker, and 3 minutes later were 50 per minute, while the blood pressure was 52 mm. and the heart rate 235. Six minutes after this, or 12 minutes after the tricresol serum injection, the blood pressure was 72 mm., the heart rate 245, and

the respirations 55. The animal was now in good condition and was later anesthetized to death. It is regretted that no attempt was made to suture the animal in order to permit it to survive. Figure 8 reproduces the striking and instructive picture which the animal furnished during the last injection with tricresol serum.

#### EXPERIMENTS ON DOGS.

The experimental results which were obtained in the dog when this animal was injected intraspinally with plain serum or with serum containing preservatives were markedly different from those observed in the monkey. The dog, under the experimental conditions previously described, was much more sensitive to the injections than the monkey.

In all the dogs except in the medulla series laminectomy was performed and the respiration and blood pressure recorded. Before describing the results obtained in the dog it must again be stated that all the dogs of the various series were anesthetized by means of intratracheal insufflation; and it will be remembered that this method at the same time furnishes an efficient artificial respiration. It is this method which explains the discrepancy between the results to be recorded and those obtained, for example, by Hale.<sup>19</sup>

*Tricresol Series.*—Six dogs were used for the intraspinal injection of 0.3 per cent. tricresol serum, and their weights ranged between 4,250 and 6,600 gm. The doses injected varied between 3 and 6 c.c. per kilo<sup>20</sup> of animal, except in one instance, dog 1, where more than 11 c.c. per kilo were given. Only one dog, No. 4, succumbed after an injection, and this animal which had already tolerated 6 c.c. per kilo probably also would have survived if the final injection of 5 c.c. had not been given while the animal was breathing spontaneously; moreover, insufflation was not begun in the attempt to resuscitate the animal until respiration had ceased for 3 minutes and was only continued for a few minutes. At the conclusion of the experiments all the dogs were breathing spontaneously when insufflation was stopped and the blood pressure of the six animals<sup>21</sup> varied between 80 and 138 mm. of mercury, the heart rate between 135 and 200, and the spontaneous respiration between 5 and 70 per minute.

The first injection of tricresol serum caused in all animals a deep drop in blood pressure which began usually before the injection was finished; this drop was occasionally preceded by a short slight rise. The level reached during this drop was often 45 mm., and was always associated with a marked slowing of

<sup>19</sup> Hale, *loc. cit.*

<sup>20</sup> These doses are not absolutely correct, because in dogs a certain variable leakage always occurred after several injections had been given intraspinally.

<sup>21</sup> In dog 4 the blood pressure was 76 mm., the spontaneous respirations 5 to 10, and the heart rate 140 per minute, before the final injection was given.

the pulse rate and an increased amplitude of the pulse beat, unless the vagi had been cut or atropin had been given. The pressure and heart rate gradually increased after the injection and within 10 minutes the former level was usually reattained. The respiration was quickened and deepened during an injection, then rapidly slowed and usually stopped in an expiratory position for a length of time which varied between a fraction of a minute and several minutes, when recovery quickly set in. Figure 9 illustrates exceptionally severe effects both upon the blood pressure and respiration after a first injection; it also shows well how complete the recovery may be after comparatively few minutes. This dog showed a drop in blood pressure from 110 to 42 mm. after 5 c.c. of 0.3 per cent. tricrosol serum, the heart rate fell from 135 to 80 per minute, and the respiration was entirely abolished for about 4 minutes. Recovery was prompt, and 12 minutes after the injection the blood pressure, heart rate, and spontaneous respirations were identical with those obtained before the injection.

Subsequent injections of tricrosol serum in dogs did not often cause more severe blood pressure depression than the first, but recovery at times became slower when 4 to 5 c.c. per kilo of animal were exceeded. A similar effect was observed with the respiration. After 5 c.c. and more per kilo had been injected, the spontaneous respirations at times reappeared more slowly and were slow and deep with inspiratory stoppages, the rate being 5 to 10 per minute; the blood pressure in this stage, however, was never lower than 80 mm. When the amount did not exceed 4 c.c. per kilo, the spontaneous respirations after recovery ranged between 35 and 70 per minute at the end of an experiment, and the blood pressure varied between 80 and 100 mm.

Figure 10 shows the respiratory slowing brought on by large doses of 0.3 per cent. tricrosol serum. This dog, No. 1, had received 30 c.c. of the serum before the injection which the curve illustrates. The next 5 c.c. of the serum, which brought the total amount to about 7 c.c. per kilo, produced only a moderate fall of blood pressure (from 98 to 70 mm.) in spite of a powerful slowing of the pulse rate (from 140 to 65) due to stimulation of the vagus center. After about 5 minutes the effects of this vagus center stimulation had largely disappeared, the blood pressure recorded 140 mm., and the heart rate was 105. The respiration which was slow before this last injection (15 per minute) was slightly stimulated at first by the injection,<sup>22</sup> and then stopped for less than 3 minutes, when a group of respirations with long inspiratory stoppages appeared; 2 minutes later spontaneous respirations were about 10 to the minute. This slowing of the respiration was not always permanent; in this same animal, for example, the next two injections of tricrosol serum were followed by a much more prompt and complete respiratory recovery than figure 10 illustrates.

The readiness and completeness with which a dog may recover from a severe effect of tricrosol serum on the blood pressure and respiration is well shown by dog 2. This animal was a female, weighing 4250 gm., and almost died from asphyxia after the first injection of 5 c.c. of 0.3 per cent. tricrosol serum, because a plugged tracheal tube had been employed through an error. Nevertheless,

<sup>22</sup> This stimulating effect was much more pronounced in subsequent injections of tricrosol serum in the same dog.



after the insertion of a new tracheal tube another injection of 5 c.c. of tricrosol serum was given 15 minutes after the first one, at a time when complete recovery from the asphyxia had not yet set in. During the injection the pressure sank gradually and the heart slowed; this slowing rapidly increased, so that in less than 30 seconds after the end of the injection no heart beats were visible on the curve and the blood pressure fell to about 6 mm. The respiration was entirely abolished by the injection. Ether was now discontinued, but insufflation was kept up and the foot of the board elevated; the air pressure in the tracheal system was also slightly increased. The heart beats reappeared less than two minutes after the injection and the blood pressure began to rise slowly. No spontaneous respirations were observed, however, until 11 minutes had elapsed; the blood pressure at this time was 40 mm. This dog received two more spinal injections at 15 minute intervals, and 13 minutes after the last injection, or about 45 minutes after the time when no heart beats were visible on the curve, the blood pressure was 80 mm., the heart rate 140, and spontaneous respirations were 35.

*Chloroform Series.*—Four dogs were used in this series; their weights were 9,000, 4,200, 4,250, and 8,000 gm., respectively. The serum injected was 0.3 per cent. chloroform serum obtained from the Massachusetts Board of Health, and the quantity used varied from 25 to 35 c.c. As usual, the dose was 5 c.c. for each injection. None of the dogs succumbed to the injection.

The effects on the blood pressure and respiration were definitely less severe after most of the injections than that exerted by tricrosol serum. Nevertheless, in each dog except No. 2 a profound drop of blood pressure occurred, which in one instance reached 70 mm. (from 123 to 54 mm.) in 2 minutes after the injection. These profound blood pressure drops usually were only observed as a result of the first injection; the succeeding injections caused only moderate depressions. Recovery resulted in most instances within 10 minutes; in the example mentioned above, where the blood pressure fell 70 mm., recovery began at once after the low level was reached, and 7 minutes later it was 112 mm.

The respiration showed the same type of effect noted in the tricrosol series. After the initial increase in rate and amplitude during the injection, the respiration slowed and stopped for a period of time ranging from a few seconds to several minutes, the length of the stoppages increasing at times with the number of injections. In only one dog, No. 2, did the animal fail to reestablish an efficient spontaneous respiration. This animal weighed about half as much as the other dogs in this series, 4,200 gm., and received in all 30 c.c. of chloroform serum. The first injection of the serum gave a profound drop in blood pressure, described above, and stopped the respiration for 4 minutes. The next three injections of 5 c.c. each stopped the respiration for not longer than 2 minutes. The fifth injection was given while the animal was breathing spontaneously and insufflation was only begun after the respiration had stopped entirely for 1.5 minutes and when the blood pressure was 36 mm. Fifteen minutes after this last injection, although the blood pressure and respiration had not yet fully recovered, a sixth injection was administered. The blood pressure dropped from 70 to 36 mm., and the respiration ceased entirely and did not return during the next hour. The blood pressure was raised by a number of adrenalin injections, but spontaneous respiration did not reappear.

The other three dogs showed at the end of the experiment a blood pressure which varied from 82 to 142 mm., pulse beats from 130 to 230, and spontaneous respirations of good amplitude from 25 to 50 per minute.

*Ether Series.*—This group consisted of four experiments. The dogs weighed 8,750, 9,200, 8,500, and 5,850 gm., respectively, and all received from 20 to 25 c.c. of horse serum to which 0.3 to 0.4 per cent. ether by volume had been added. The serum was not warmed before injection. None of the dogs succumbed.

The effect on the blood pressure and respiration was in general much less than that exerted by either the chloroform or tricresol serum. The drop in blood pressure was slight to moderate, usually associated with a slowing of the pulse rate (vagus pulses) unless the vagi were cut; recovery was very swift and a normal level was reattained after a minute or two. In many instances the drop in blood pressure in spite of a strong slowing was quite slight. The respiration was also only slightly affected after the large majority of the injections, and the respiratory stoppages when they occurred did not last longer than a fraction of a minute.

A serious effect was obtained only once and this occurred in dog 1, a female of 8,750 gm. Both vagi were cut in this animal before any injections were given, and the respirations were in consequence slower than normal. The first two injections of 5 c.c. each of 0.3 per cent. ether serum, separated from each other by a 15 minute interval, caused practically no effect on the respiration or blood pressure. Now the intratracheal insufflation was discontinued and the animal was allowed to breathe spontaneously through the tracheal catheter. Then the third injection was given intraspinally. The respiration first slowed strongly and then stopped entirely before the injection was finished. The blood pressure after an initial rise dropped abruptly from 128 to 38 mm. within 45 seconds after the end of the injection. Before this low level was quite reached, spontaneous respiration began again after a stoppage in expiration of about 45 seconds; both the respiration and blood pressure rapidly became better, but after 4 minutes the respiration again slowed and now showed long inspiratory stoppages. At this point intratracheal insufflation was reestablished and ether was discontinued. After 8 minutes conditions were worse, the blood pressure was lower, and the respirations slower. 1 c.c. of adrenalin in 3 c.c. of Ringer solution was injected intraspinally. The blood pressure and respirations improved at once, and 11 minutes later the blood pressure registered 108 mm., the pulse beats were 265, and the respirations were 25.

One hour after this injection, which produced such a deep effect, two more injections of ether serum were given. These two injections, separated by 15 minutes, caused only slight effects on the blood pressure and respiration. At the end of the experiment the animal had a blood pressure of 86 mm., pulse of 220, and the respirations numbered 35 per minute.

*Horse Serum Series.*—In this series sterile horse serum without any preservative was injected intraspinally. Five experiments were made, but in three of these either tricresol or chloroform serum was injected before plain serum was used. Because these later injections produced well marked effects on the blood pressure and respiration, the combination experiments were rejected because the results could not definitely be attributed to the plain horse serum.

In two experiments nothing but sterile horse serum, at least a month old

and without any preservative, was injected intraspinally. The dogs weighed 5,250 and 6,000 gm., and both were males. The first dog received 30 c.c. and the second one 35 c.c. of sterile serum intraspinally, and both were in good condition at the end of the experiment. Dog 1 (vagi intact) at that time had a blood pressure of 80 mm., pulse rate of 165, and 15 respirations per minute. Dog 2 (vagi cut) registered a pressure of 76 mm., a pulse rate of 175, and a respiration of 55 per minute.

Every injection in dog 1, whose vagi were intact, caused a drop in blood pressure varying from 20 to 50 mm., and the pulse was at the same time increased in amplitude and slowed in rate (vagus pulses). The drop was usually preceded by a short moderate rise of pressure. The drop usually lasted as long as the slowing of the pulse was marked, but four times out of the six injections in this dog the original level was reattained at a time when the pulse rate was still 15 to 40 beats slower than normal. The duration of the drop varied from a fraction of a minute to 4 minutes.

In dog 2, where the vagi were cut, the injection of plain horse serum produced a drop of blood pressure which usually did not exceed 15 mm. This drop established itself more slowly, and in addition was preceded by a more sustained rise than in dog 1 (vagi intact). During the low blood pressure level the rate was usually slowed 10 to 20 beats, although the vagi were sectioned in this animal. The duration of the drop was usually less than 1 minute.

One severe drop of blood pressure was obtained in dog 2. The seventh injection of 5 c.c. of plain serum with no preservative was given 20 minutes after the sixth dose, while the animal was breathing spontaneously; before the injection was completed the blood pressure fell from 106 to 64 mm. in less than one minute, and three minutes later reached the 50 mm. level, the pulse slowing from 175 to 165. Then recovery set in without any insufflation or other aid, and 12 minutes after the lowest level was reached (17 minutes after the injection) the blood pressure recorded 92 mm. The respiration throughout this time was spontaneous and showed no marked alterations of any kind. It should be added that the next injection which was given 7 minutes after the last one produced only the ordinary effect.

The respiration in dog 1 (vagi intact) showed the usual initial quickening and deepening of the respiration during the injection, followed by a slowing with inspiratory prolongation and a stoppage of respiration in the expiratory position which lasted from a half minute to 2 minutes. The respirations were at first slow but the normal rate and depth were usually reattained quickly. In dog 2 (vagi cut) inspiratory stoppage of the respiration was the most pronounced phenomenon during the injection, and the following respiratory stoppage in expiration did not exceed 1 minute in duration.

Both dogs received plain horse serum intraspinally while breathing spontaneously and the effect was apparently no more severe in all, except one instance, than when given during artificial respiration. This single and striking exception occurred in dog 2, and has already been described.

*Ringer Solution Series.*—Two male dogs, weighing 7,500 and 7,000 gm., respectively, were used and both received 9 injections of 5 c.c. of Ringer solution intraspinally. The vagi of both were intact. The injections were given at 7 minute intervals, sometimes even more rapidly. The effects on the blood

pressure and respiration were in general similar to those described for the ether or plain horse serum series,—initial rise of the blood pressure followed by a slowing of the pulse rate and a moderate drop of pressure which usually did not exceed 20 mm. Within one minute the normal level and pulse rate were reattained. The respiration often was merely slowed, and when stoppage in expiration took place, this stoppage never lasted longer than 1 minute.

Both animals were in good condition at the end of the experiment and each tolerated two injections of 5 c.c. of tricrosol serum with considerably less effect on the blood pressure and respiration than that serum usually produced; the blood pressure sank about 20 mm, with a moderate slowing of the rate, and recovered its level within a few minutes. The respiration was not stopped at all in any of the four tricrosol injections. Ten minutes after the last injection both dogs showed blood pressure 110, 108 mm., pulse rate 195, 175, and respiration 30, 55, respectively.

*Formalin Experiments.*—A few experiments in dogs only were made with a serum to which formalin in the proportion of 1 or 2 per 1,000 serum had been added. With 1 to 1,000 the effect on the blood pressure and respiration was considerably less severe than with 0.3 per cent. tricrosol. This investigation was not continued because formalin is a powerful protein coagulant and there is at present no evidence available that the antimenigitis serum is not injured by this preservative.

*Local Applications of Tricrosol Solutions to the Medulla.*—This series of experiments was carried out in order to observe the effect of 0.3 per cent. tricrosol when applied directly to the fourth ventricle. A procedure of this kind reduces the factor of pressure of the tricrosol solution so that it can be neglected, and the effects observed may safely be attributed to the tricrosol solution itself.

Ten experiments were carried out in dogs. In the first four experiments 0.3 per cent. tricrosol in 0.9 per cent. salt solution was employed. This solution was applied to the medulla at about 5 minute intervals while the animal was breathing spontaneously or during intratracheal insufflation. In none of these four tests did the saline tricrosol solution cause a marked lowering of the blood pressure or a stoppage of the respiration. When ether was pushed so that the spontaneous respiration began to fail and the blood pressure fell, the tricrosol saline hastened the disappearance of respiration. If the tricrosol application was regularly continued, but the ether stopped while air insufflation was maintained for about 5 minutes longer before this also was discontinued, then spontaneous respirations began almost at once, and the blood pressure continued its ascent which began as soon as the ether was stopped.

The last six experiments of this series were made by applying 0.3 per cent. tricrosol horse serum to the medulla. Before each application the cerebrospinal liquid was cautiously removed with absorbent cotton and replaced by 0.6 to 1 c.c. of tricrosol serum. The blood pressure and respiration were recorded as usual. In five of the six dogs one vagus was cut in the neck and the central stump was used for stimulation of the medullary centers. Five of these dogs weighed between 6 and 10 kilos; only one weighed less (dog 5).

Four of the six experiments showed that several applications of 0.3 per cent. tricrosol serum in 7 to 10 minute intervals produced only slight alterations of the blood pressure and respirations; the respirations usually became more rapid

and of smaller amplitude; the blood pressure sank about 10 mm.; both respiration and blood pressure became practically normal within a few minutes. At the end of these four experiments a molecular solution of magnesium sulphate was applied to the medulla: in each dog the respiration promptly slowed and stopped permanently, the blood pressure fell gradually, and the animal died without a struggle.

In two experiments, dogs 6 and 8, the medullary application of 0.3 per cent. tricrosol serum caused a respiratory stoppage. In dog 6 two applications each of 0.6 c.c. of tricrosol serum caused first a short increase in the rate coupled with a diminished amplitude of the respirations, then the rate slowed and respirations ceased for less than a minute when they again appeared, although the tricrosol solution had not been removed. Removal of the tricrosol solution and the application of Ringer solution hastened the recovery. The blood pressure was not affected at all. In this experiment the intratracheal insufflation was continued throughout.

In dog 8 two applications each of 1 c.c. of 0.3 per cent. tricrosol serum to the medulla caused a stoppage of the respiration in the expiratory position after a short period of respiratory stimulation of the rate, as in dog 6. During this stoppage the intratracheal insufflation was discontinued 1 minute after the practical abolition of respiration, but nevertheless spontaneous respirations began 1 minute later and rapidly became normal in amplitude, though faster in rate. It must be noted that the tricrosol solution was not removed, but was allowed to remain in contact with the medulla. The blood pressure showed a drop of 13 mm. Ten minutes after the first application, the solution was removed and replaced by another c.c. of tricrosol serum. The blood pressure was at this time 92 mm., the pulse rate 150, and the respirations numbered 60 per minute. The application again caused a stoppage of spontaneous respirations which lasted about 2 minutes when spontaneous respirations appeared; the insufflation of air was stopped 30 seconds after the beginning of respiratory stoppage. The blood pressure in this instance dropped slowly from 88 to 79 mm. Seven minutes after the application the blood pressure was 100 mm., the pulse 165, and the spontaneous respirations were 70 per minute. In this test tricrosol serum was again allowed to remain in contact with the medulla throughout and again complete recovery resulted, without aiding the animal in any way. Figure 11 shows the effect produced by the first application of 0.3 per cent. tricrosol serum on the medulla of dog 8 of this series, which thus produces quite different results from the intraspinal injection of the same liquid.

#### DISCUSSION.

From the description of experiments given in the preceding pages a number of facts appear with clearness. It has been shown that monkeys tolerate the intraspinal injection of 0.3 per cent. tricrosol serum very well, even when previously subjected to an operation which interferes to some extent with respiration (partial pneumothorax). Out of eight monkeys only one succumbed, and this one

died after receiving about 3 c.c. per kilo of body-weight. The reasons for this death will be considered later. The other monkeys survived the injections well, though the doses given were often considerably greater than the one which caused the single death of this series. Indeed, the quantities tolerated under unfavorable conditions by the monkeys were a source of surprise. Among the operated monkeys of this series (pleural cannula, spontaneous respiration, cannula in the femoral artery, ether anesthesia throughout) two monkeys were used on successive days for the same experiment, the injections on the second day being given while the animal had a full one-sided pneumothorax. Monkey 4 received 18 c.c. of 0.3 per cent. tricresol serum intraspinally (5.2 c.c. per kilo) in six injections at fifteen minute intervals, and was in excellent condition at the end of the experiment. On the next day the same animal, in apparently excellent condition, was again injected with 23 c.c. of 0.3 per cent. tricresol serum (8.2 c.c. per kilo) with full recovery. Figures 1 and 2 show the effects of the first and sixth injection on the first day; and figure 3 shows the result of the seventh injection, this last time 5 c.c., on the next day. The intervals were again fifteen minutes. Similar results were obtained with monkey 6 which was operated in the same manner as monkey 4. Monkey 6 received on the first day 15 c.c. of tricresol serum in five injections at fifteen minute intervals, and was in excellent condition at the end. The wounds were then treated with tincture of iodine and sutured as usual. No attempt was made to remove the pneumothorax which resulted on removal of the pleural cannula. On the next day the animal seemed to be in good condition and was again prepared for a graphic record of the respiration and blood pressure. On this day it received intraspinally in 3 c.c. doses at fifteen minute intervals 18 c.c. of tricresol serum (6 c.c. per kilo) without any alarming symptoms. Then 5 c.c. of Ringer solution were injected as fast as possible, but the animal recovered within a few minutes. Another monkey, No. 7, received 21 c.c. of tricresol serum intraspinally (8 c.c. per kilo) in 3 c.c. doses at seven minute intervals, and was in excellent condition at the end of the experiment and on the next day, in spite of a one-sided pneumothorax. Four days after the injection, however, the animal died of dysentery which it had con-

tracted before the injection, for five of its normal mates died of the same disease.

These experiments show decisively that large quantities of 0.3 per cent. tricrosol antimeningitis serum are tolerated very well by monkeys which have been subjected to severe operations. But these experiments may be considered acute and it might be suggested that the result would be different if the injections had been repeated a number of times on different days and the animals then observed for a period of days. Experiments of this type were, therefore, also carried out. Monkeys 1, 2, and 3 received intraspinal injections of 2 to 3 c.c. of 0.3 per cent. tricrosol serum per kilo on different days without any anesthesia, and were then kept under observation for at least one month. All of them were alive and in excellent condition after that period. Monkey 1 was injected on five different days, monkey 2 on four, and monkey 3 on two days. Only one of these monkeys showed an alarming collapse after an injection, but recovered promptly after a period of pharyngeal insufflation.<sup>23</sup>

These experiments show that repeated injections of fairly large amounts of 0.3 per cent. tricrosol serum do not cause undesirable effects which appear only after some days.

It must be emphasized that all the monkeys received intraspinal injections without any artificial respiratory aid.

In the tricrosol dog series similar results were obtained. Out of six dogs which received from 3 to 6 c.c. of 0.3 per cent. tricrosol serum per kilo of body-weight, one dog succumbed after having tolerated 6 c.c. per kilo. This dog died after the next injection of 5 c.c., apparently because this last injection was given without intratracheal insufflation while the animal was breathing spontaneously. If this last injection had also been given during intratracheal insufflation it seems probable that the animal would have survived like his companions.

These results in dogs are at variance with those observed by Hale,<sup>24</sup> who obtained a fatal issue when "somewhat larger" doses

<sup>23</sup> Collapse after the mere introduction of a hypodermic needle into the spinal dural sac was also observed in this series. It is possible that a preceding partial asphyxia due to the method of holding these lively animals is partly responsible for the collapse.

<sup>24</sup> Hale, *loc. cit.*, p. 14.

than 2 c.c. per kilo were injected intraspinally. This difference is entirely due to the different methods which Hale employed, for in his experiments the dogs were allowed to breathe spontaneously, while in my experiments almost invariably intratracheal insufflation was used which prevents asphyxia due to any temporary functional failure of the respiratory center.

*Sources of Danger from 0.3 Per Cent. Tricresol Serum.*—One of the main dangers in the employment of 0.3 per cent. tricresol serum seems to be the increased intraspinal pressure. This is indicated quite clearly in a number of experiments both in the dog and in the monkey. Indeed, in the monkey the death-producing factor may be largely the increased intraspinal pressure. In the monkey, for example, the injection of a few cubic centimeters of tricresol serum after the previous intraspinal introduction of plain horse serum without any preservative may produce a stoppage of the respiration broken occasionally by short gasps, a profound drop in blood pressure, and a marked slowing of the pulse. Nevertheless, the mere removal of spinal fluid may suffice to restore blood pressure, respiration, and pulse to normal within a few minutes. An experiment of this type is well shown in figure 8.<sup>25</sup>

In this animal a similar serious effect in the respiration and blood pressure had been obtained before on the same day and the monkey had rallied with similar speed after some spinal fluid was withdrawn, though this time it was also aided by pharyngeal insufflation. This respiratory aid was probably not necessary, for the second respiratory failure yielded promptly when merely spinal fluid was withdrawn, as shown in figure 8.

The single death in the tricresol monkey series (animal 5) gave a tracing of the respiration and blood pressure after the fatal injection which is practically identical with that in figure 8. In this fatal case pharyngeal insufflation was administered, but no spinal fluid was withdrawn. It seems probable that the issue would have been different had some spinal fluid been allowed to escape.

In the dog, also, pressure plays a considerable part. This is shown by the differences in effect between intraspinal injections of

<sup>25</sup> The blood pressure reached its normal level a few minutes later on the tracing following the one printed in figure 8.



tricrosol serum and its local application to the medulla. In this animal species the intraspinal injection of 0.3 per cent. tricrosol usually causes a powerful drop in blood pressure associated with a marked slowing of the pulse and a more or less marked abolition of respiration (figures 9 and 10). But the local application of the same tricrosol solution on the medulla of the dog only exceptionally (in two dogs out of ten) produces a pronounced stoppage of the respiration (figure 11), and never, in my experience, a strong, continued drop of blood pressure with a slowing of the pulse. Here again the only difference seems to be one of pressure: there is practically none when the tricrosol solution is applied locally, but when an injection is given intraspinally there must be a rise of intraspinal pressure until the injected liquid is absorbed. It is possible that the condition of the medullary centers previous to the injection of tricrosol serum is of importance. Monkey 5 of the tricrosol series, for example, had been almost killed by an excess of ether during the operation, and the experiment was carried out before it had recovered completely. This animal succumbed after receiving only 9 c.c. of 0.3 per cent. tricrosol serum intraspinally. The symptoms were practically identical with those observed in another monkey, used for the second time, when large doses of serum, both normal and tricrosol, had been injected. In this latter monkey it will be remembered that the symptoms were promptly abolished by the mere withdrawal of some spinal fluid (figure 8).

These experiments show that the stress laid by Flexner<sup>26</sup> on the part of increased intraspinal pressure in the production of death in human beings after tricrosol meningitis serum is experimentally well founded.

Although increased intraspinal pressure apparently suffices to explain in large part the causation of dangerous symptoms in the monkey, and the differences observed in the dog when tricrosol serum is applied locally to the medulla or injected intraspinally, nevertheless there is evidence that the tricrosol itself exerts an effect. This is indicated by the stronger blood pressure effect obtained in monkeys after tricrosol serum than after chloroform, ether, or plain horse serum. In the dog also the same difference between the various

<sup>26</sup> Flexner, *loc. cit.*

sera is observed, tricrosol serum usually causing a more profound blood pressure drop than the other sera. In addition, the dog, but not the monkey, usually exhibits a severer interference with the respiration after tricrosol serum than after plain serum. Moreover, as mentioned previously, the mere, local application of 0.3 per cent. tricrosol serum on the medulla of the dog may occasionally produce a transient stoppage of the respiration. All these facts indicate that 0.3 per cent. tricrosol does affect the medullary centers, but the action is definitely much more pronounced in the dog than in the monkey. The nature of these medullary effects will be discussed in a following section.

Another source of danger, which was observed only in unanesthetized monkeys, is the production of a collapse by the introduction of a needle into the subdural space of the spinal cord. This collapse is usually marked by a slowing and weakening of the respiration, which may be so great that artificial respiration (pharyngeal insufflation) is necessary to save the animal. These effects were obtained four times in two monkeys of the three in this series, and occurred after the animal had already tolerated several injections of tricrosol serum. The collapse could not be obtained at will, and these and the remaining animal gave no alarming reactions when the spinal dural sac was punctured repeatedly in order to bring on a collapse. It seems probable that a preceding partial asphyxia is an important factor in producing this type of collapse. The two monkeys which showed it struggled violently while being held across the knee of the assistant. In order to immobilize them sufficiently to permit an intraspinal insertion of the needle, the chest and abdomen had to be pressed firmly across the knee, and this must have interfered with respiration. In addition to this, the asphyxia may have led to an increased intraspinal pressure, for Dixon and Halliburton<sup>27</sup> have demonstrated that asphyxia, however produced, always leads to a considerable increase in the secretion of the cerebrospinal liquid.

<sup>27</sup> Dixon and Halliburton, *loc. cit.*, p. 234.

## ANALYSIS OF THE EFFECT OF INCREASED INTRASPINAL PRESSURE AND TRICRESOL ON THE MEDULLARY CENTERS.

The chief danger which results to the organism when the intraspinal pressure is raised by tricresol serum is an interference with efficient respiration due to an action upon the respiratory centers. This action was not great in most of the monkey experiments and large quantities of 0.3 per cent. tricresol serum injected intraspinally by syringe produced usually but slight effects on the respiration (figures 2, 3, and 4). Nevertheless, a serious stoppage may be obtained now and then, as shown in figure 8 where the respiration practically ceased until the intraspinal pressure was reduced. In dogs, however, temporary cessation of respiration is a fairly constant accompaniment of most intraspinal injections of 0.3 per cent. tricresol serum, and this abolition of respiration often lasted some minutes even though intratracheal insufflation was maintained throughout the period of cessation of spontaneous respiration. The respiratory center of the dog is thus much more sensitive to 0.3 per cent. tricresol serum than that of the monkey. The same fact is indicated by the ease with which respiratory stoppages are obtained in the dog after the injection of sera with and without preservatives or even by Ringer solution alone. In the monkey, on the other hand, no marked respiratory effects are usually obtained with any serum beyond those which occur when the injection is driven in rapidly (compare figures 4 and 7), especially when the animal is not completely under anesthesia.

This respiratory failure in the dog after tricresol injections has been interpreted by Hale<sup>28</sup> as a paralysis of the respiratory center due to tricresol, although he admits that increased intracerebral pressure plays some part. It has already been shown that increased intraspinal pressure plays a very important part in the causation of serious symptoms both in the monkey and dog, and it must now be determined whether all the available facts support Hale's assumption that the respiratory center is really paralyzed in the dog after large doses of tricresol serum.

The analysis of the effects observed after the intraspinal injection of tricresol serum, or any fluid, is made difficult by the fact that the

<sup>28</sup> Hale, *loc. cit.*, pp. 14 and 18.

functional alterations observed are or may be the result of antagonistic actions upon the medullary centers. Thus a stimulation, either inhibitory or excitatory, of both the vagus and vasomotor centers must neutralize each other to some extent, though both centers may be clearly recognized at work in the blood pressure curve in some instances (figure 10); or the respiratory center may show both stimulation of inspiration as well as a stimulation of inhibition of inspiration. On the other hand, however, it can easily be determined whether or not a center has been paralyzed, for the function presided over by that center must then disappear permanently, or at least the recovery must be very slow. It will be shown in the following that the changes produced by 0.3 per cent. tricresol serum injected intraspinally can readily be explained as the result of central stimulation, either excitatory or inhibitory, and that a paralysis can be excluded.

When 5 c.c. of 0.3 per cent. tricresol serum are injected into the spinal dural sac of the dog for the first time, the usual effect is a respiratory stoppage in passive expiration which persists for a variable length of time. The recovery is usually complete within a few minutes. Here obviously a paralysis of the respiratory center cannot be assumed; the recovery is too prompt (figure 9). Moreover, after doses of 0.3 per cent. tricresol serum, so large that with Hale's method the dog would surely succumb, for example with 6 c.c. per kilo, the respirations are still good and each may show an inspiratory tetanus (figure 10), which of course means a stimulation of inspiration. If another injection of tricresol serum is then given intraspinally the respirations stop now in passive expiration for a variable number of minutes (inhibition of inspiration) and then respirations of the same inspiratory type as before the injection appear (figure 10). The stoppage in expiration must therefore be interpreted, it seems to me, as an inhibition of the respiratory center and not as a paralysis. This supposition is strengthened by observations which show that an intraspinal injection, given while an animal shows shallow, slow respirations due to preceding tricresol injections, nevertheless brings out a short series of rapid respirations of large amplitude. Here again the facts speak definitely for inhibition and stimulation of the respiratory center and not for a paralysis, as it is

obviously impossible for a paralyzed structure to send out a series of strong normal impulses.

This distinction between an inhibition and an intoxication or paralysis of a structure is not academic, in spite of the fact that the results of both may be disastrous to the animal. If a center is merely inhibited, no damage to the structure itself is necessarily implied; there is merely a stoppage of function, the cells themselves being intact; they will function as soon as the inhibition wears off sufficiently, and the organism will survive if tided over the critical period. If, however, a center is paralyzed, this means a damage to the cells of that center, and recovery can take place only after this damage has been at least partly repaired, which is necessarily a slow process. If, therefore, a vital center is inhibited there is the probability that the center will again resume its activity if given sufficient time, and if its function is in the meantime replaced. When the center inhibited is the respiratory center, this is very easily and readily done by instituting adequate artificial respiration. It is for this reason that the tricrosol dog series reported in this paper showed such excellent results after the injection of large doses of tricrosol serum; all the dogs were injected while intra-tracheal insufflation was maintained except in one dog, No. 4, weighing 4,750 grams, which succumbed after having tolerated 30 c.c. of 0.3 per cent. tricrosol serum, apparently because the last injection was given while the animal was breathing spontaneously.

The stimulation of excitatory and inhibitory functions by 0.3 per cent. tricrosol serum is not limited to the respiratory center of the dog; it is also clearly exhibited by the vagus and vasomotor centers of the medulla. Almost any injection of 5 c.c. of 0.3 per cent. tricrosol serum in the dog brings on a definite slowing of the pulse rate which is often very pronounced for some time. The pulse rate, for example, may drop from 135 to 80 per minute, or from 140 to 65 (figures 9 and 10). This means undoubtedly that the vagus center has been stimulated by the tricrosol serum, for section of the vagi or atropin abolishes the great slowing of the pulse. The vasomotor center of the dog also exhibits reactions which are most readily explained by a stimulation or inhibition. The first injection of tricrosol serum in a dog usually causes a drop in blood pressure

and this drop may be profound; it may fall from 110 to 42 mm. (figure 9). This drop, however, can only be partly explained as a result of vagus center stimulation, for a drop will still occur if the vagi have been sectioned. The tricrosol serum, therefore, must have exerted an effect on the vasomotor center, which may either have been an inhibition of the normal vasomotor tonus or a stimulation of the vasodilating center. An intoxication or paralysis can be excluded by the swift recovery which occurred within twelve minutes in an unusually severe case (figure 9). Moreover, the behavior of the center during subsequent injections of tricrosol serum shows clearly that intoxication and paralysis can be excluded. After several injections of 0.3 per cent. tricrosol serum a stage may be reached where the stimulation of the vagus center produced by an injection becomes very pronounced, and where, in spite of the considerable slowing of the pulse rate, the blood pressure rises so that it may greatly exceed the normal level. Here evidently both the vagus and the vasoconstrictor centers are stimulated at the same time. Such a combined stimulation of vagus and vasoconstrictor centers is shown in figure 10. This tracing was obtained from a dog of 8,750 grams, which had already received 6 c.c. of 0.3 per cent. tricrosol serum per kilo before the injection was given which the curve illustrates. From these data it is therefore permissible to conclude that the vasomotor center also is not intoxicated or paralyzed, but is, on the contrary, stimulated by large doses of 0.3 per cent. tricrosol serum. Attention may be called here to a statement of Hale,<sup>29</sup> that the "blood pressure always falls" after the intraspinal injection of tricrosol serum in the dog. This I have found by no means true in my experiments. Figure 10, for example, shows only a moderate drop of the mean blood pressure, and in subsequent injections of tricrosol serum in the same animal I have obtained tremendous rises in blood pressure with stoppage of the respiration in expiration quite comparable to Hale's figure,<sup>30</sup> illustrating a fatal rise of intraspinal pressure after the injection of plain serum without any preservative. This tracing of Hale shows very well a stoppage of respiration in the expiratory position and a stimulation of both the vagus and vasomotor

<sup>29</sup> Hale, *loc. cit.*, p. 17. This statement is italicized in the original.

<sup>30</sup> Hale, *loc. cit.*, p. 13.

centers resulting in a strong rise of blood pressure, although the pulse slows from 300 to 192 per minute. I have obtained similar results after large doses of 0.3 per cent. tricresol serum with this difference, that the tricresol dog survived.

From the behavior of the respiratory, vagus, and vasomotor centers of the dog after large doses of 0.3 per cent. tricresol serum have been injected intraspinally, the inference may thus justly be drawn that the effects observed are the results of a stimulation either excitatory or inhibitory of these centers. These effects, however, may be disastrous to the dog, for they interfere with an efficient respiration, and the combination of cessation of respiration together with a low blood pressure will sooner or later be fatal to the dog, because a vicious circle is formed, unless artificial respiration is maintained for some time after each injection.

A low blood pressure in itself after 0.3 per cent. tricresol serum does not seem to be dangerous to the life of the animal, provided that respiration is maintained either spontaneously or artificially. This has been observed both in the monkey and the dog. Plate 10 shows a prompt recovery of the blood pressure after a drop of 71 mm. of mercury; in dog 2 of the same series the blood pressure fell abruptly to practically zero after 19 c.c. of 0.3 per cent. tricresol serum intraspinally, and the curve showed no heart beats. Nevertheless, intratracheal insufflation was maintained and the animal not only recovered but tolerated two further injections each of 5 c.c. of tricresol serum. Twelve minutes after the last injection the blood pressure was 80 mm., the pulse rate 140, and the spontaneous respirations 35 per minute.

In the monkey, also, low blood pressure after tricresol serum does not seem to threaten life. For example, figure 3 shows a drop from 94 to 43 mm. after the intraspinal injection of 5 c.c. of 0.3 per cent. tricresol serum. Recovery took place within ten minutes without any aid. The respiration was not much affected beyond a transitory quickening of the rate and a slight diminution of the amplitude of each respiration.<sup>31</sup>

<sup>31</sup> This monkey, it must be pointed out, had already received 18 c.c. of 0.3 per cent. tricresol serum before the 5 c.c. which the figure shows. Moreover, the same animal had been used the day before for the same type of experiment and had then tolerated easily 18 c.c. of 0.3 per cent. tricresol serum intraspinally. The monkey weighed 3,453 gm.

Another marked drop of blood pressure in the monkey is recorded in figure 6 after 5 c.c. of 0.3 per cent. tricresol serum. This animal had already received 18 c.c. of 0.3 per cent. chloroform serum and 6 c.c. of tricresol serum. The dose now injected caused a drop from 76 to 38 mm.; spontaneous recovery resulted within six minutes. The respirations in this animal also were quickened and slightly reduced in amplitude, but never showed any signs of failing. The most severe blood pressure drop in the monkey is shown in figure 8. In this animal the respiration failed and the blood pressure dropped to the 18 mm. level. Nevertheless, prompt recovery both of blood pressure and respiration resulted as soon as the intraspinal pressure was reduced by allowing the spinal fluid to escape through the hypodermic needle.

These results concerning the comparative harmlessness of considerable drops of blood pressure both in the dog and monkey after intraspinal injection of tricresol serum, unless associated with respiratory failure of some degree, do not invalidate the observation of Sophian<sup>32</sup> that the blood pressure drop is an accurate guide in judging what quantity of serum can safely be injected intraspinally in the human being. Every danger signal in the human subject must be observed during a therapeutic intervention, and as the blood pressure has shown itself a good index in the hands of Sophian, it should continue to be used when serum is injected intraspinally in the human being. It is, of course, evident, even without the work reported in this paper, that the respiration also should be watched as carefully as the blood pressure, and injection should be discontinued temporarily as soon as the respiration shows alterations.

#### COMPARISON OF SERA EMPLOYED.

From the description already given of the effects exerted by the various sera used in this investigation, it is seen that they can be arranged in order of their effect on the blood pressure and respiration. In the dog the greatest action on the blood pressure and respiration was exerted by 0.3 per cent. tricresol serum; 0.3 per cent. chloroform serum produces also a well marked blood pressure drop and stoppage of the respiration, but the severity of these changes

<sup>32</sup> Sophian, A., *Jour. Am. Med. Assn.*, 1912, lviii, 843.



is almost always much less than with the tricresol serum; the difference is especially outspoken in the effect on the respiration, the stoppages being almost always less than those obtained with the tricresol serum. The action of 0.3 per cent. ether serum is still less than that of the chloroform serum, and the respiration and blood pressure are usually only moderately affected. Plain horse serum is perhaps somewhat less active in its effect on the blood pressure and respiration than the ether serum, though the difference is not pronounced.

None of the sera tested in the dog were without some adverse action both on the respiration and the blood pressure. Plain horse serum caused at times respiratory stoppages in passive expiration which lasted longer than a minute, and in one instance after an injection of 3.5 c.c. of plain serum while the dog (vagi cut) was breathing without any aid, a prompt, profound drop of blood pressure from 106 to 50 mm. occurred while the rate merely slowed from 175 to 165, the respirations remaining unaltered at 45 per minute. Full recovery took place within 20 minutes. This effect was probably due to an inhibition of the tonus of the vasomotor center, and was quite similar to the drop of blood pressure obtained after 0.3 per cent. tricresol serum.

In the monkey the same gradation of the sera holds in general, except that the effects of all are strongly less than those observed in the dog. 0.3 per cent. tricresol serum, for example, only exceptionally produces a marked effect on the respiration even after large doses have been injected intraspinally (figures 2, 3, 4), and no marked respiratory effects were observed at all after chloroform, ether, and plain serum. The difference between the sera is best emphasized in the monkey by the behavior of the blood pressure: 0.3 per cent. tricresol serum causes usually a more or less pronounced drop of blood pressure (figures 1, 2, 3, 4); 0.3 per cent. chloroform serum has much less effect (figures 5, 6); 0.3 per cent. ether serum causes no drop of blood pressure, but raises the pressure for a short period of time (figure 7); and plain horse serum also practically does not affect the blood pressure, the rise being less than with ether serum.

These experiments show that ether and chloroform sera are better

than tricresol, if we consider the blood pressure and respiration as criteria. Moreover, they have this advantage, that they can be largely if not entirely removed by warming the serum container before injection. The preservative thus may fulfill its function as long as it is considered desirable.

*Effect on the Spinal Cord.*—The spinal cord was usually examined both in dogs and monkeys during the autopsy after an experiment. In the dog the blood vessels of the cord were usually found moderately injected after 0.3 per cent. tricresol serum, especially in the lumbar and lower thoracic region. At times the cord at the site of injection showed some maceration of the surface, but only in one instance were hemorrhages found in the substance of the cord itself, and these hemorrhages were limited to the lumbar region. Similar injections of the pia-arachnoid vessels were observed after the employment of the other sera, but the injection was usually less. Superficial macerations of the cord were also observed in these series.

In the monkey the cord was examined shortly after an experiment or after an interval of days or weeks. No damage to the cord was ever seen, and the injection of the blood vessels was never pronounced even after 0.3 per cent. tricresol serum. In no instance were adhesions noted between the cord and dura, nor did the cord show gross signs of infection in spite of the fact that the antisepsis practiced during an intraspinal injection was far from perfect.

It was interesting to note that even after very large doses of serum the monkey's spinal dural sac did not contain a large amount of fluid, even when the autopsy was performed less than 30 minutes after the last injection. Absorption of the serum seems to be rapid, and this fact explains the rapidity with which the blood clotted in the arterial cannula and adjoining section of artery during blood pressure experiments.

*Effect of Preservatives on the Opsonins of Antimeningitis Serum.*—This series of experiments was kindly carried out by Dr. Martha Wollstein. The results show that 0.3 per cent. tricresol, 0.3 per cent. chloroform, or 0.3 per cent. ether affect the opsonin content about equally when tested after one week, one month, and three months.

#### SUMMARY.

The monkey (*Macacus rhesus*) usually tolerates readily the repeated intraspinal injection of large doses of 0.3 per cent. tricresol antimeningitis serum. The spontaneous respiration is generally not disturbed.

Doses of 0.3 per cent. tricresol serum as large as 8 c.c. per kilo were injected intraspinally with subsequent recovery, even when the monkey had a partial pneumothorax.

Dangerous alterations of the respiration and blood pressure in the monkey after 0.3 per cent. tricresol serum given by syringe are

apparently largely due to increased intraspinal pressure, for the mere reduction of this pressure has sufficed to bring about a prompt and complete recovery.

The medullary centers of the monkey (vagus, respiratory, and vasomotor) are highly resistant to the action of sera when injected intraspinally, strikingly more so than those of the dog.

Occasionally the mere introduction of a hypodermic needle into the spinal dural sac of non-anesthetized, unoperated monkeys which have already received injections of 0.3 per cent. tricresol serum, may produce a severe collapse. A preceding partial asphyxia seems to be a necessary condition.

Large quantities of sera are rapidly absorbed from the spinal dural sac of monkeys, and the clotting time of the blood is decreased.

The spinal meninges of the monkey are resistant to infection; even primitive precautions during intraspinal injections apparently suffice to prevent infection.

Dogs are much more sensitive to the intraspinal injection of 0.3 per cent. tricresol serum than monkeys; nevertheless they may tolerate as much as 6 c.c. per kilo provided that intratracheal insufflation is maintained for some time after each injection.

The chief danger in dogs after intraspinal injections of 0.3 per cent. tricresol is a cessation of the respiration; for this reason artificial respiration is necessary.

The blood pressure in the dog may be profoundly lowered by 0.3 per cent. tricresol serum, yet recovery is usually obtained if intratracheal insufflation is maintained.

The effects of 0.3 per cent. tricresol serum upon the medullary centers is interpreted to be the result of either excitatory or inhibitory stimuli. No evidence was found that either the respiratory, vasomotor, or vagus center is paralyzed.

The local application of 0.3 per cent. tricresol serum upon the exposed medulla of dogs does not produce the same effect upon the respiration and blood pressure as intraspinal injection of the same serum.

A solution of 0.3 per cent. tricresol serum applied locally to the medulla of dogs occasionally produces a transient respiratory stop-

page, without markedly affecting the blood pressure even when intratracheal insufflation is stopped.

Increased intraspinal pressure was found to be an important factor in the production of respiratory and blood pressure changes in the dog after intraspinal injection of 0.3 per cent. tricresol serum.

Both in the monkey and in the dog 0.3 per cent. chloroform serum, 0.3 per cent. ether serum, or plain horse serum produced in general a smaller effect upon the medullary centers than 0.3 per cent. tricresol serum.

The ideal preservative for therapeutic sera would seem to be one which could be removed before injection. Ether in this respect is better than chloroform.

The opsonins in antimeningitis serum are about equally affected by 0.3 per cent. tricresol, 0.3 per cent. chloroform, or 0.3 per cent. ether when tested after one week, one month, and three months.

When intraspinal injections are given in the human being it would seem advisable to be prepared to withdraw part of the injected fluid and to administer artificial respiration, if necessary. For a safe withdrawal of fluid the gravity method is the best; for artificial respiration Meltzer's apparatus for pharyngeal insufflation is recommended.

*Addendum.*—After this paper was completed and ready for press an article appeared by Voegtlin<sup>33</sup> which demands consideration. Voegtlin's object was to study the quantitative effects of various tricresol serum concentrations on the blood pressure and respiration when injected intraspinally in dogs and monkeys by the gravity method or by syringe; also to investigate the comparative effects of normal serum and of sera preserved by phenol, tricresol, formalin, or chloroform, after intraspinal injection.

Voegtlin avoided the use of non-volatile anesthetics of the type employed by Hale in order to exclude their depressant effects upon the medullary centers; he substituted warm ether vapors which were administered to the animals through a tracheotomy wound. The respirations were recorded from the pleural cavity by means of a cannula and a Marey tambour. The blood pressure was taken from the carotid artery and a mercury manometer was used. The intraspinal injections in the monkey were given in the usual way, that is, by introducing a needle through the skin and between the laminæ of the upper lumbar spinal column into the dural sac. In dogs, however, Voegtlin did not expose the cord by resecting the laminæ of the lumbar spinal column, a procedure which both Hale and I found necessary to secure uniform results, but he inserted the needle between two vertebræ after the spinal column was freely exposed. After obtain-

<sup>33</sup> Voegtlin, C., *Bull. Hyg. Lab., U. S. P. H. and M.-H. S.*, 1914, No. 96, 87.

ing spinal fluid, the needle was connected with a syringe or a burette according to the method employed for injection. When the gravity method was used a pressure of 40 to 50 cm. was found satisfactory.<sup>34</sup>

From his investigation, largely confined to a study of the blood pressure, Voegtlin concludes that the effect on the blood pressure increases in proportion to the rate of injection and the concentration of the phenol preservative;<sup>35</sup> that there is no difference between the toxicity of phenol and tricresol serum of the same concentration;<sup>36</sup> that the fall of blood pressure is primarily due to the inherent toxic action of the phenol or tricresol preservative on the medullary centers, and not to increased intracranial tension, because the fall increases with an increase of the concentration.<sup>37</sup> He suggests chloroform as a preservative for antimeningitis serum, because its intraspinal injection produced no dangerous effects.<sup>38</sup> He also recommends the use of the gravity method.

The experimental results which Voegtlin obtained with 0.3 per cent. tricresol serum, chloroform serum, or normal horse serum are in accord with those which my experiments have yielded, at least as far as they are shown by his illustrative tables and protocols. Indeed, his results with 0.3 per cent. tricresol serum and with chloroform serum were better than those which I obtained. With 0.3 per cent. tricresol serum Voegtlin reports not a single death in either dogs or monkeys; the lowest level of the blood pressure reached after a 0.3 per cent. tricresol injection was 55 mm. (monkey 4, gravity method)<sup>39</sup>, and the recovery of the blood pressure was good in all recorded instances. The respiratory effect of 0.3 per cent. tricresol in dogs seems to have been slight; a respiratory stoppage is nowhere noted in the protocols, and only once is the respiration described as depressed (dog 6, gravity method).<sup>40</sup> In spite of these good results recorded in his protocols from the tricresol serum, he states<sup>41</sup> that with 0.3 and 0.5 per cent. tricresol serum the respiration very often stopped after a dose of 3 to 5 c.c., the blood pressure fell to a low level, and this was followed by the death of the animal in a few minutes, unless artificial respiration was given. The protocols and tables, which Voegtlin must have considered typical because he incorporated them in his article, do not support the above statement in its entirety, but only as far as 0.5 per cent. tricresol serum is concerned. All the deaths in the protocols dealing with 0.3 per cent. tricresol were caused by the subsequent injection of 0.5 per cent. tricresol and never by 0.3 per cent. tricresol. As all my work with tricresol serum was limited to the 0.3 per cent. strength, which is the quantity used in practice as a serum preservative, I am unable to discuss the effect of the 0.5 per cent. strength.

The effect on the blood pressure caused by tricresol serum administered by the gravity method he interprets as primarily due to the inherent toxic property

<sup>34</sup> Voegtlin, *loc. cit.*, p. 89.

<sup>35</sup> Voegtlin, *loc. cit.*, p. 118.

<sup>36</sup> Voegtlin, *loc. cit.*, p. 116.

<sup>37</sup> Voegtlin, *loc. cit.*, p. 116.

<sup>38</sup> Voegtlin, *loc. cit.*, p. 118.

<sup>39</sup> Voegtlin, *loc. cit.*, p. 110.

<sup>40</sup> Voegtlin, *loc. cit.*, p. 92.

<sup>41</sup> Voegtlin, *loc. cit.*, p. 115.

of this preservative and not to the increased intracranial tension.<sup>42</sup> While I am far from denying that 0.3 per cent. tricrosol serum *per se* exerts an effect on the medulla, for my own experiments with local application of this solution to the medulla of the dog definitely show a reaction, nevertheless I must point out that the experiments exhibit a distinct difference between local medullary applications and intraspinal injections, even if given by the gravity method. I have shown that the local application of 0.3 per cent. tricrosol serum to the medulla, a procedure which practically rules out the element of spinal or medullary pressure, does not produce any marked effect on the blood pressure, even when spontaneous respiration is abolished, as exceptionally happens (figure 11); yet the intraspinal injection of 0.3 per cent. tricrosol serum even by the gravity method always produces a well marked drop in blood pressure (Voegtlin).<sup>43</sup> These differences show definitely that increased intraspinal pressure is a significant factor in the production of blood pressure and respiratory effects after the intraspinal injection of 0.3 per cent. tricrosol serum.

It must be emphasized in this connection that the gravity method is no guarantee that the intraspinal pressure is not raised by its employment, unless the rate of intraspinal absorption is as great as the rate of infusion—a condition which is probably never fulfilled. The pressure in the gravity system is thus always greater than that in the dural sac of the cord, for otherwise no serum would flow in. The degree of pressure used in the gravity method depends entirely upon the operator; Voegtlin, for example, probably used a pressure of 40 to 50 cm. of serum.<sup>44</sup> Such a pressure equals about 30+ mm. of mercury, which cannot be considered negligible, for he notes the appearance of pressure symptoms when normal serum was injected by this method.<sup>45</sup> That pressure plays an important part in the grave accidents which sometimes occur after 0.3 per cent. tricrosol is well shown in figure 8 of the present article where the mere removal of some cerebrospinal liquid sufficed to restore respiration and blood pressure in a monkey. For these reasons I cannot agree with Voegtlin when he states<sup>46</sup> that he has shown conclusively that if a low rate of injection is used (gravity method) no pressure effects can be obtained with 0.3 per cent. tricrosol, although his results with this concentration warrant the belief that the pressure factor has been reduced by the gravity procedure.

Voegtlin always refers to the action of tricrosol as toxic or depressant and apparently means that this substance damages the medullary centers,<sup>47</sup> but he gives no definite proof. In the body of the present paper I have advanced reasons why this view is probably not correct, but that, on the contrary, the tricrosol, at least in 0.3 per cent. strength, acts as a stimulation to the excitatory and inhibitory functions of the medullary centers.

Voegtlin's results with the intraspinal injection of chloroform serum in the dog by the gravity method were better than those that I obtained by the

<sup>42</sup> Voegtlin, *loc. cit.*, p. 116.

<sup>43</sup> Voegtlin, *loc. cit.*, pp. 92 and 93.

<sup>44</sup> Voegtlin, *loc. cit.*, p. 89.

<sup>45</sup> Voegtlin, *loc. cit.*, p. 114.

<sup>46</sup> Voegtlin, *loc. cit.*, p. 119.

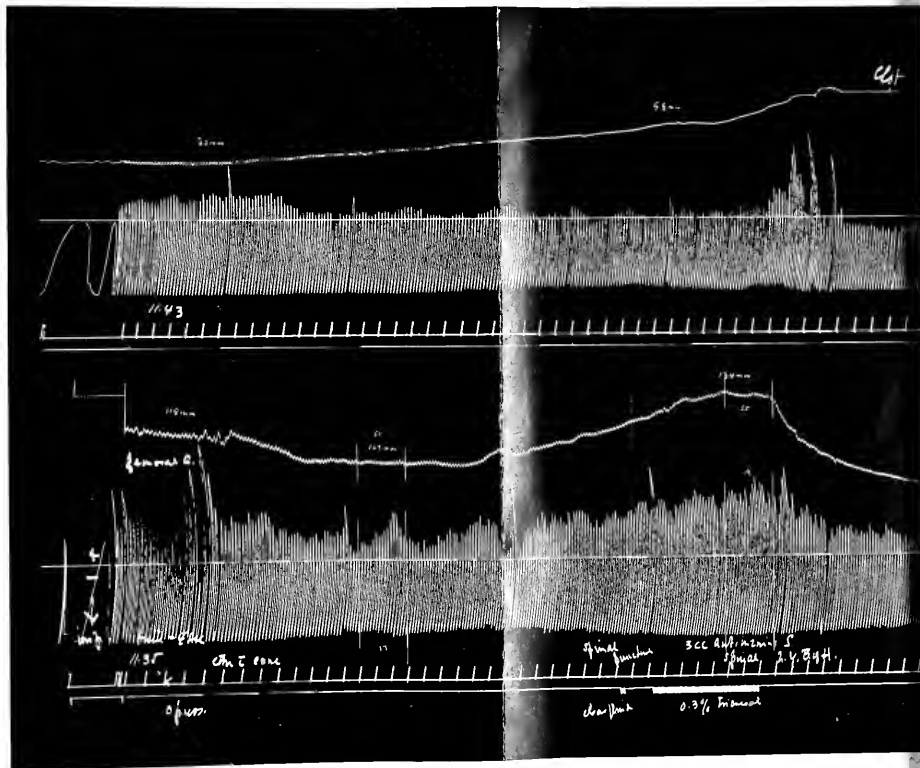
<sup>47</sup> Voegtlin, *loc. cit.*, p. 115.

## EXPLANATION OF PLATES.

In stroke stoppage. The respiratory curve is usually cut by a straight horizontal line which represents pressure; above the line is positive pressure; below the line is negative pressure. The blood pressure, obtained in the dog from the carotid artery and in the monkey from the femoral artery, is recorded by a mercury manometer. Zero level of the pressure is recorded by a horizontal line. The time line is marked in 4 second intervals. The white hands below the time line record the time consumed during the injection. In 12 second intervals have been measured, and the blood pressure, number of respirations for this interval noted in small figures. The blood pressure readings represent the mean blood pressure. The monkeys received artificial respiration as a routine procedure.

## PLATE 1.

FIGURE 1. Monkey 4, tricrosol series, weight 3.455 gm.; normal respiration; vagi intact. The figure shows the effect of 3 c.c. of 0.3 per cent tricrosol antimeningitis serum injected intraspinally for the first time. In this monkey spinal puncture was performed while the blood pressure and respiration were being recorded. Note the moderate effect of the injection on the respiration, and the prompt recovery of the blood pressure. Compare this figure with figure 6, which shows the profound effect of a first injection of 0.3 per cent tricrosol serum in the dog.



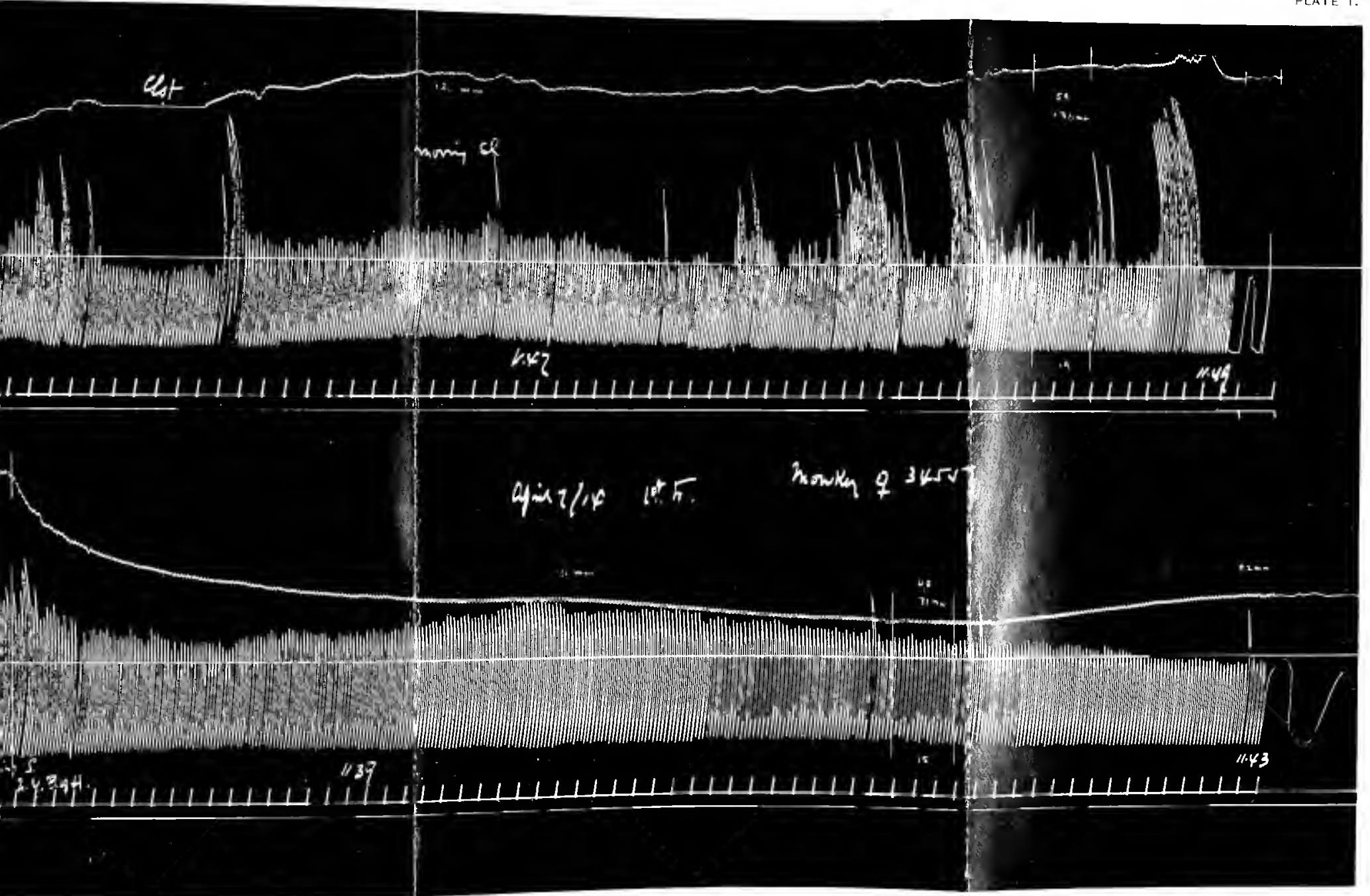


FIG. 1

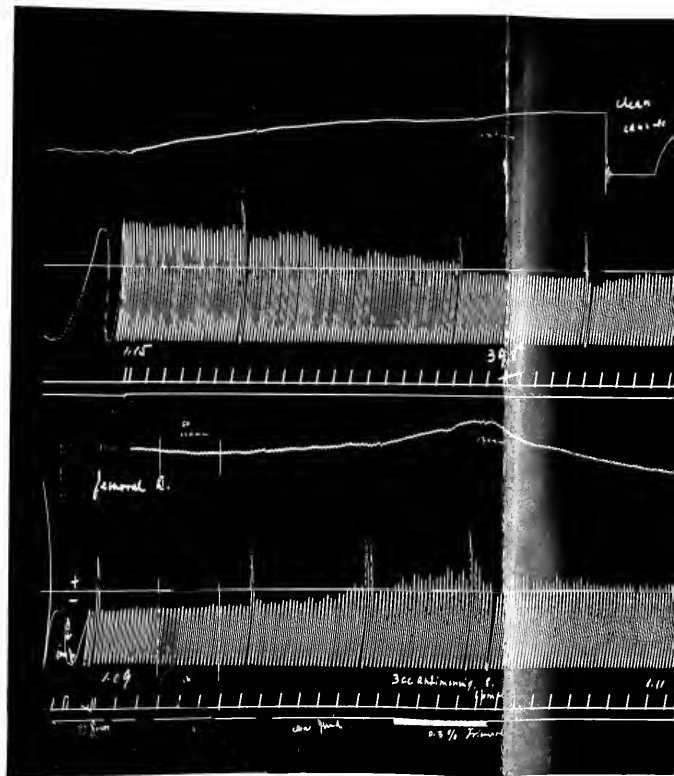
NOTE.—Intraspinal Injections of Serum.



PLATE 2.

FIG. 2. Same animal as in figure 1; same day, later stage.

The tracing shows the effect of the sixth injection of 3 c.c. of 0.3 per cent tricrosolium intraspinally. The effect on the respiration and blood pressure is less than that recorded after the first injection. 18 c.c. of 0.3 per cent tricrosolium, or 5.4 c.c. per kilo, were thus readily tolerated. The speed of the drum was increased periodically to facilitate counting the respirations and pulse beats. The wounds were now sutured, after treating them with tincture of iodine, and the animal was returned to its cage in excellent condition although it had a left pneumothorax.



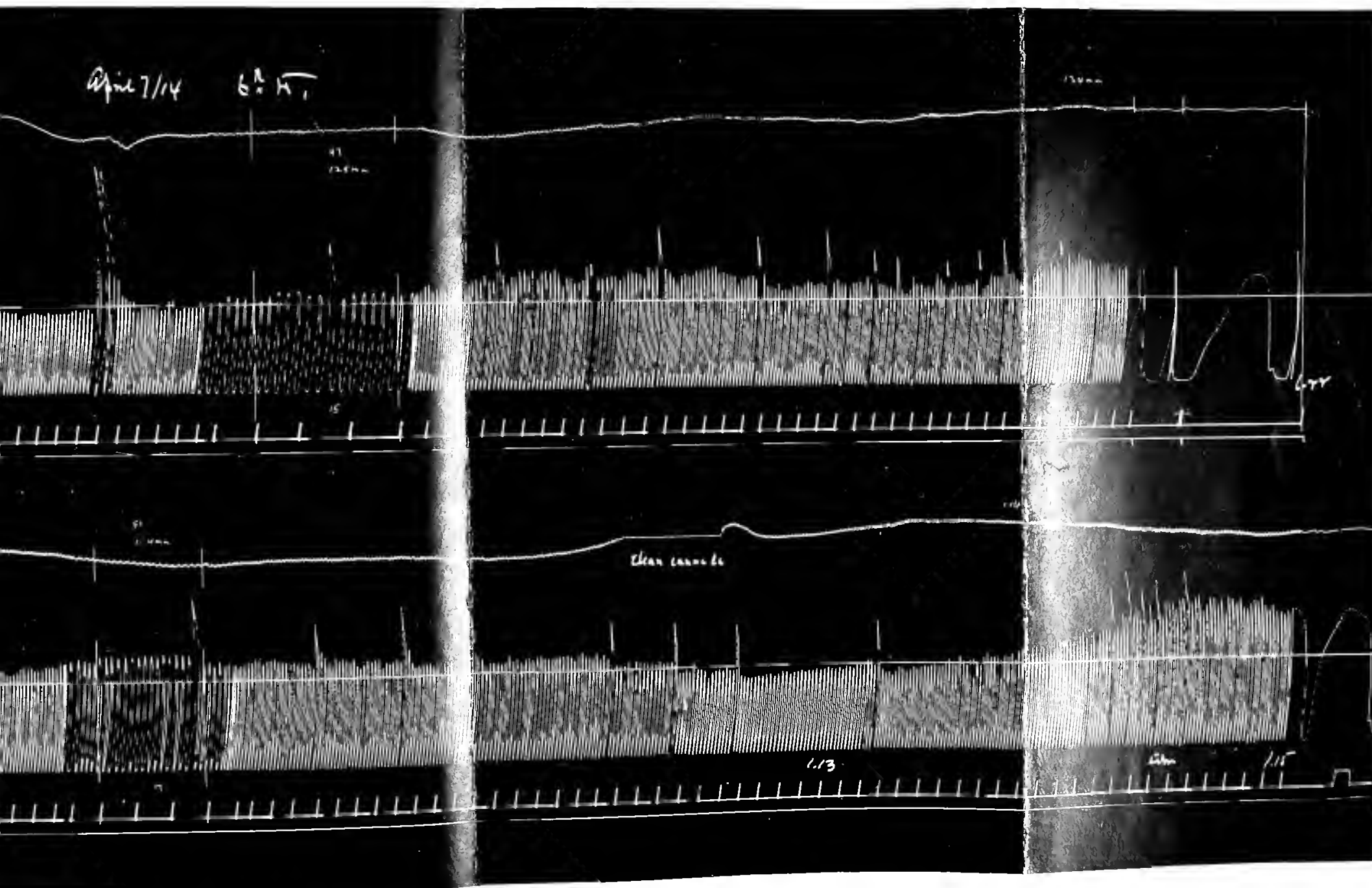
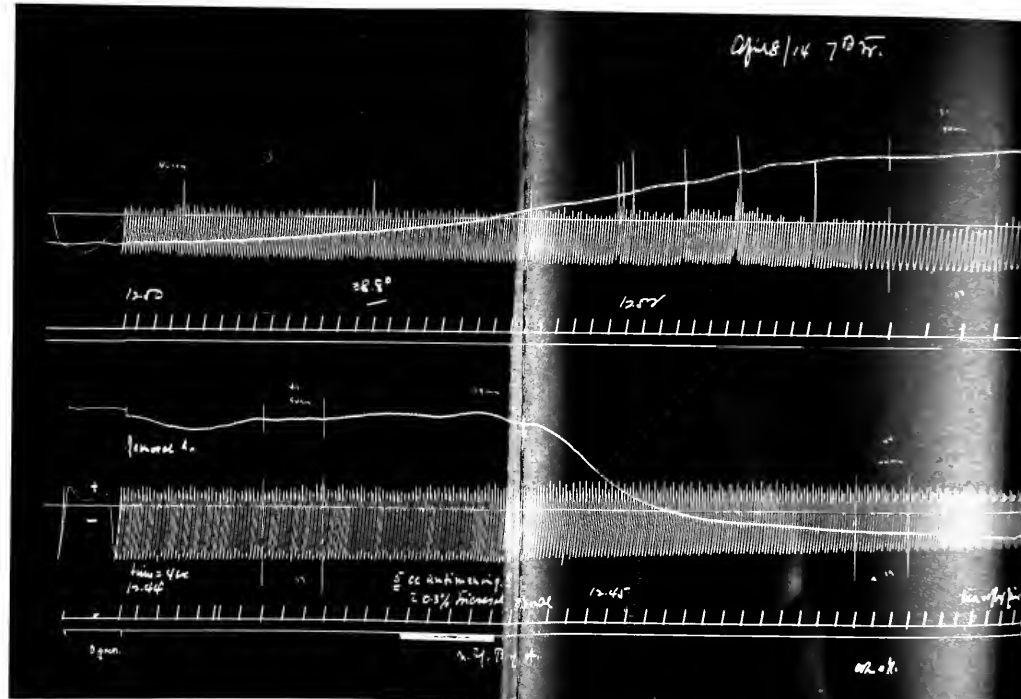


FIG. 2

A. Original Direction of Seism.

PLATE 3.

FIG. 3. Same animal as in figures 1 and 2; *near day*, vagi intact, left pneumothorax. The monkey was in excellent condition. The figure shows the effect of the seventh injection of 0.3 per cent tricrosol serum. The previous doses were 3 c.c. at 15 minute intervals, 18 c.c. in *toto*. The figure illustrates the effect of 5 c.c. injected as the usual dose of 3 c.c. Note the profound drop in blood pressure from 138 mm. accompanied by a slowing of the pulse from 245 to 215 per minute. The respirations are quickened from 85 to 105 per minute with a slight increase of the respiratory amplitude. Prompt recovery of the blood pressure occurred. This figure shows the harmlessness of a deep fall of blood pressure when the respiration is normal. Only one of the six injections preceding the figure gave a similar drop of blood pressure. Fifteen minutes after the 5 c.c. injection (figure 3) another injection of 5 c.c. of tricrosol serum was given. The curve was practically identical with figure 3; recovery of the blood pressure was well begun after 7 minutes, but was accelerated needlessly by the intraspinal injection of 2 c.c. of adrenalin which raised the blood pressure in 1 minute to 200 mm. The animal was in excellent condition at the end of the experiment and had tolerated 28 c.c. of 0.3 per cent tricrosol serum, 8.2 c.c. per kilo, without respiratory difficulty at any time.



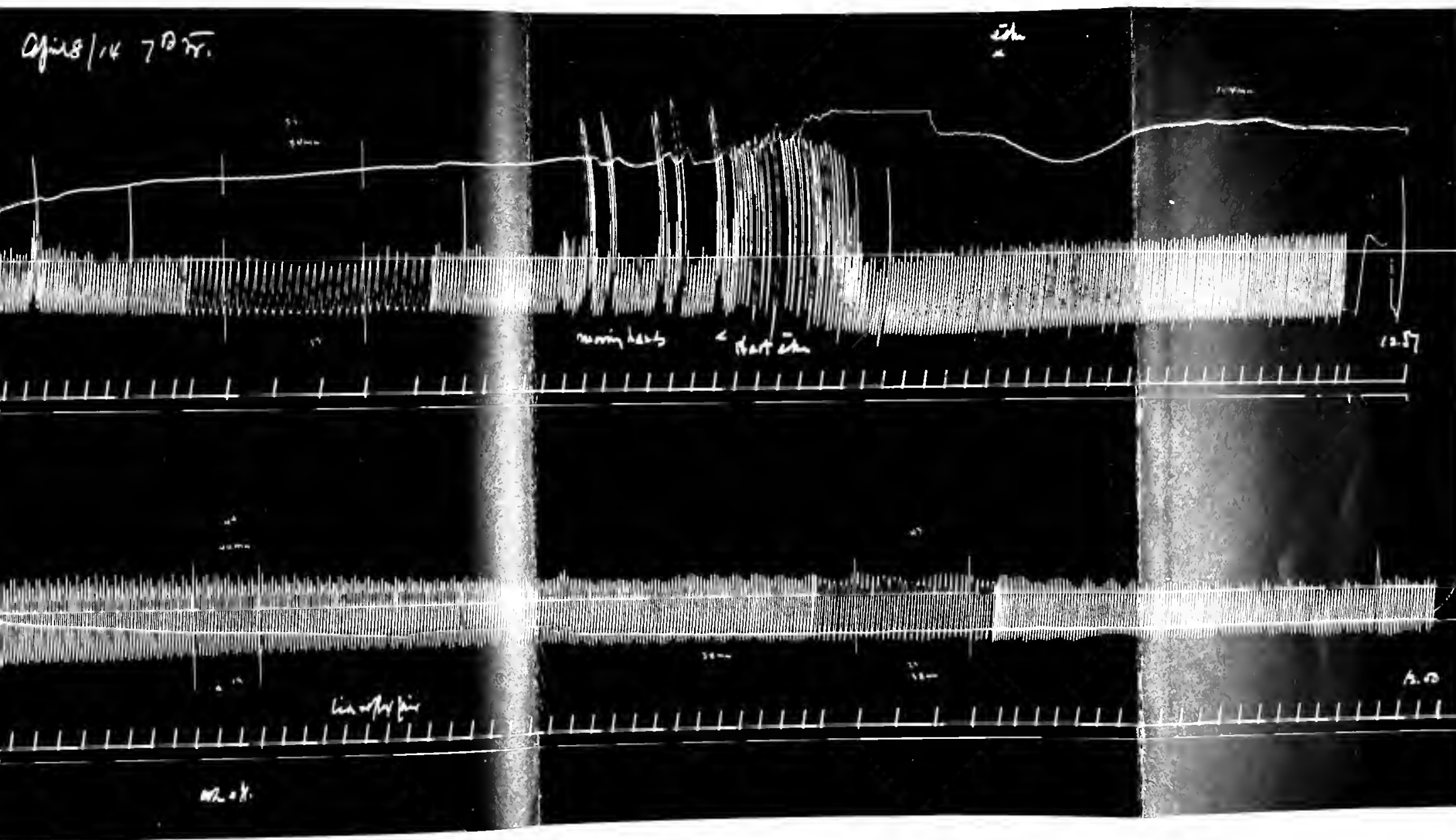


FIG. 3

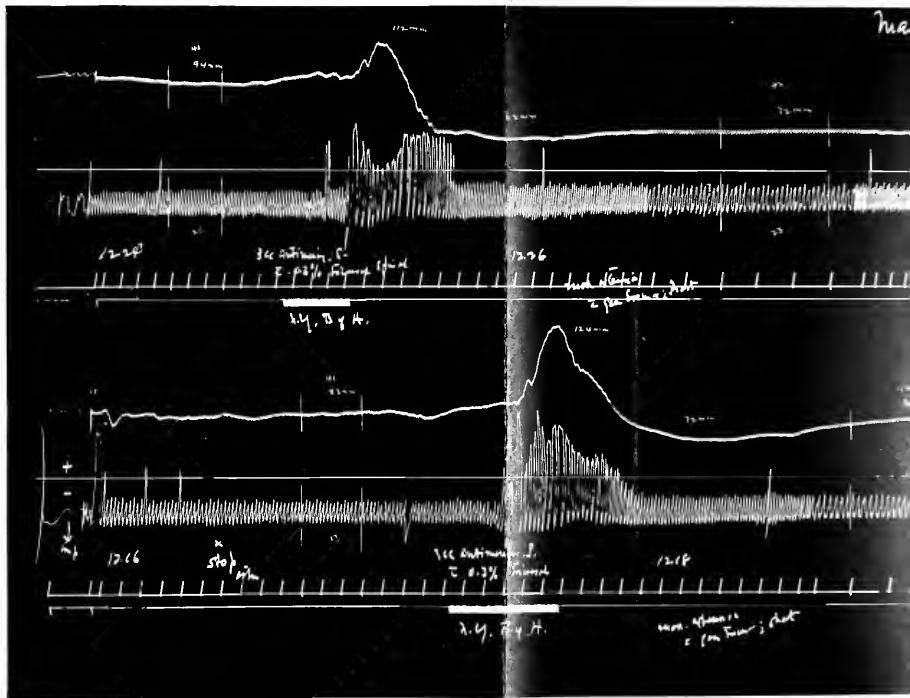
John Auer.

PLATE 4

Fig. 1. Monkey 7, tricrosol series; weight 2,865 gm.; vagi intact; normal respiration.

The tracing shows the seventh and eighth intraspinal injections of 3 c.c. of 0.3 per cent. tricrosol serum. All the injections were given at 7 minute intervals. The effect on the blood pressure was slight, beyond the short initial rise. The respiration showed an increase in rate without any marked diminution of amplitude. The initial respiratory effect of the injections (increase in amplitude with short and stoppages in active expiration) was largely due to the fact that the animal was not fully anesthetized. The animal was in excellent condition at the end of the experiment after 8 c.c. of 0.3 per cent. tricrosol serum per kilo and died the next 2 days. Four days later it died of dysentery contracted before the experiment.

THE JOURNAL OF EXPERIMENTAL MEDICINE VOL. XXI.



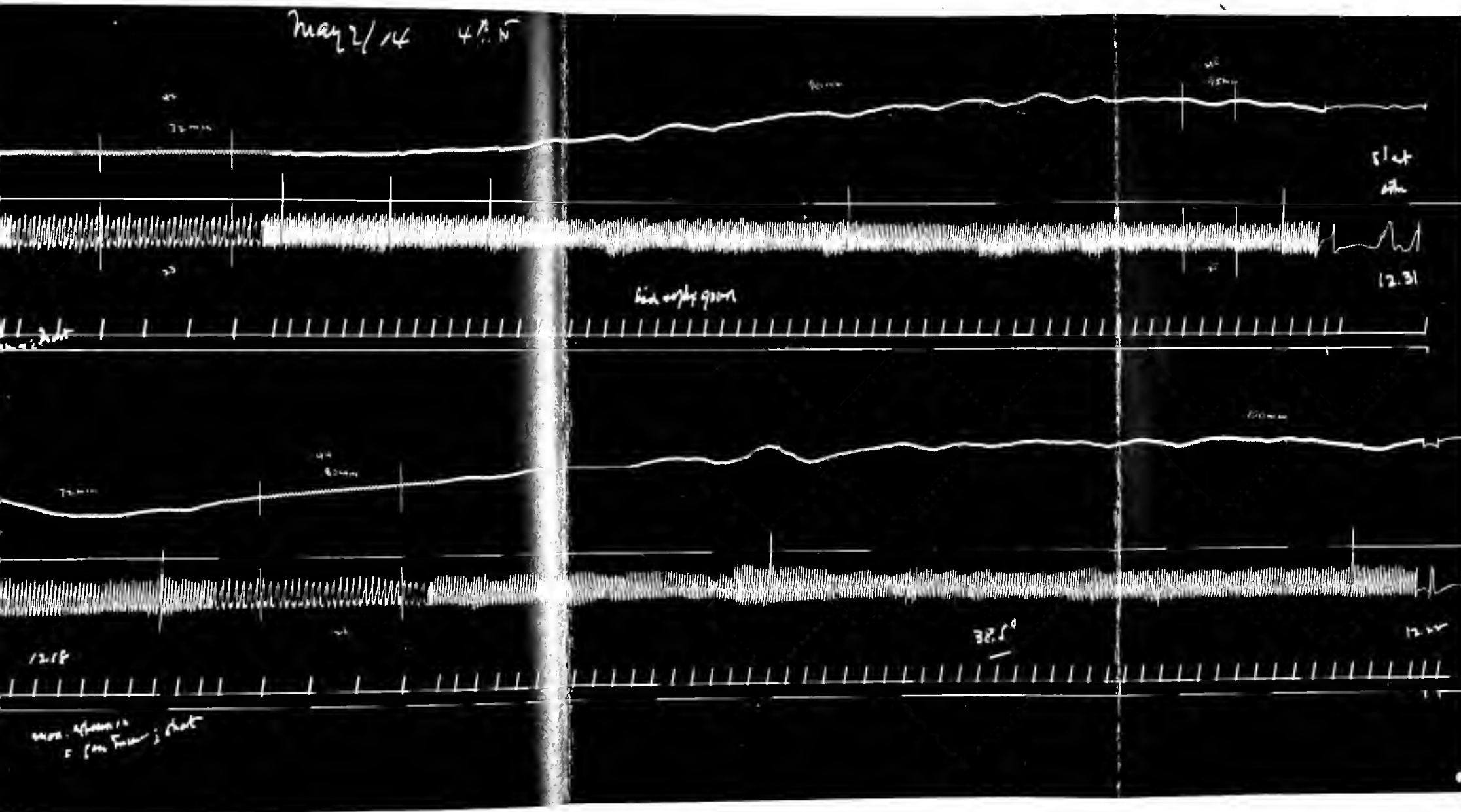


FIG. 4.

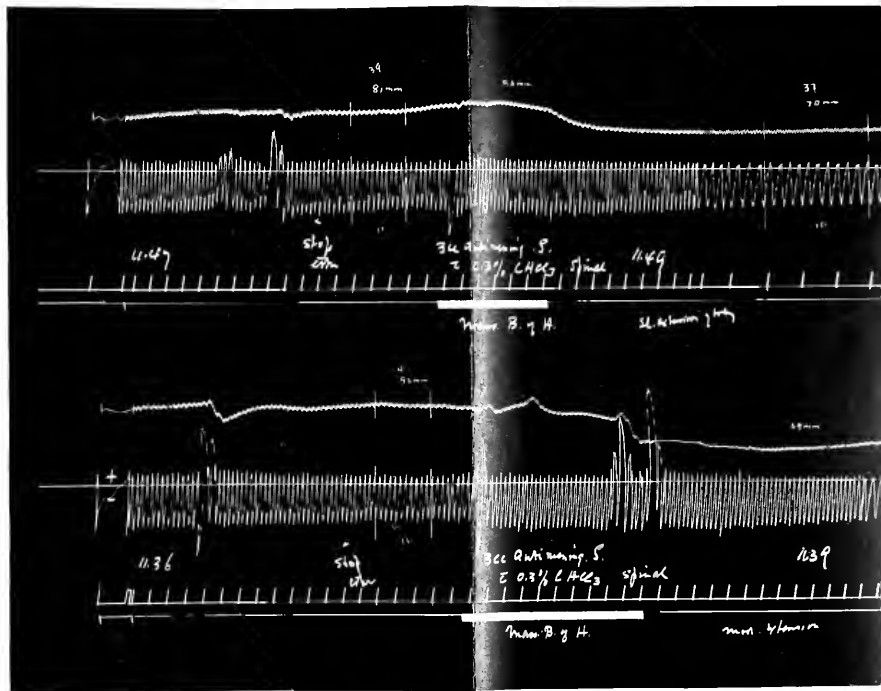
Aver. Intraspinal Functions of S. 14.1

PLATE 5

FIG. 5. May 2 (chloroform series); weight 2835 gm; vagi intact; normal respiration; pneumothorax.

The animal was received intraspinally on the day before (May 3) 15 c.c. of 0.3 per cent chloroform antineurotoxic serum and 6 c.c. of 0.3 per cent trichloroform. The blood pressure and respiration had been recorded. The interval between the injections varied between 7 and 15 minutes. After the experiment the animal was returned in excellent condition to its cage, although it had a left pneumothorax. On the next day (May 6) it was again prepared for recording while in excellent condition.

The tracing shows the slight effect exerted by two intraspinal injections each of 3 c.c. of 0.3 per cent chloroform serum, after the animal had already tolerated 3 c.c. of 0.3 per cent trichloroform serum and 6 c.c. of 0.3 per cent chloroform serum. The preceding chloroform serum injections produced about the same effect as those pictured in figure 5.



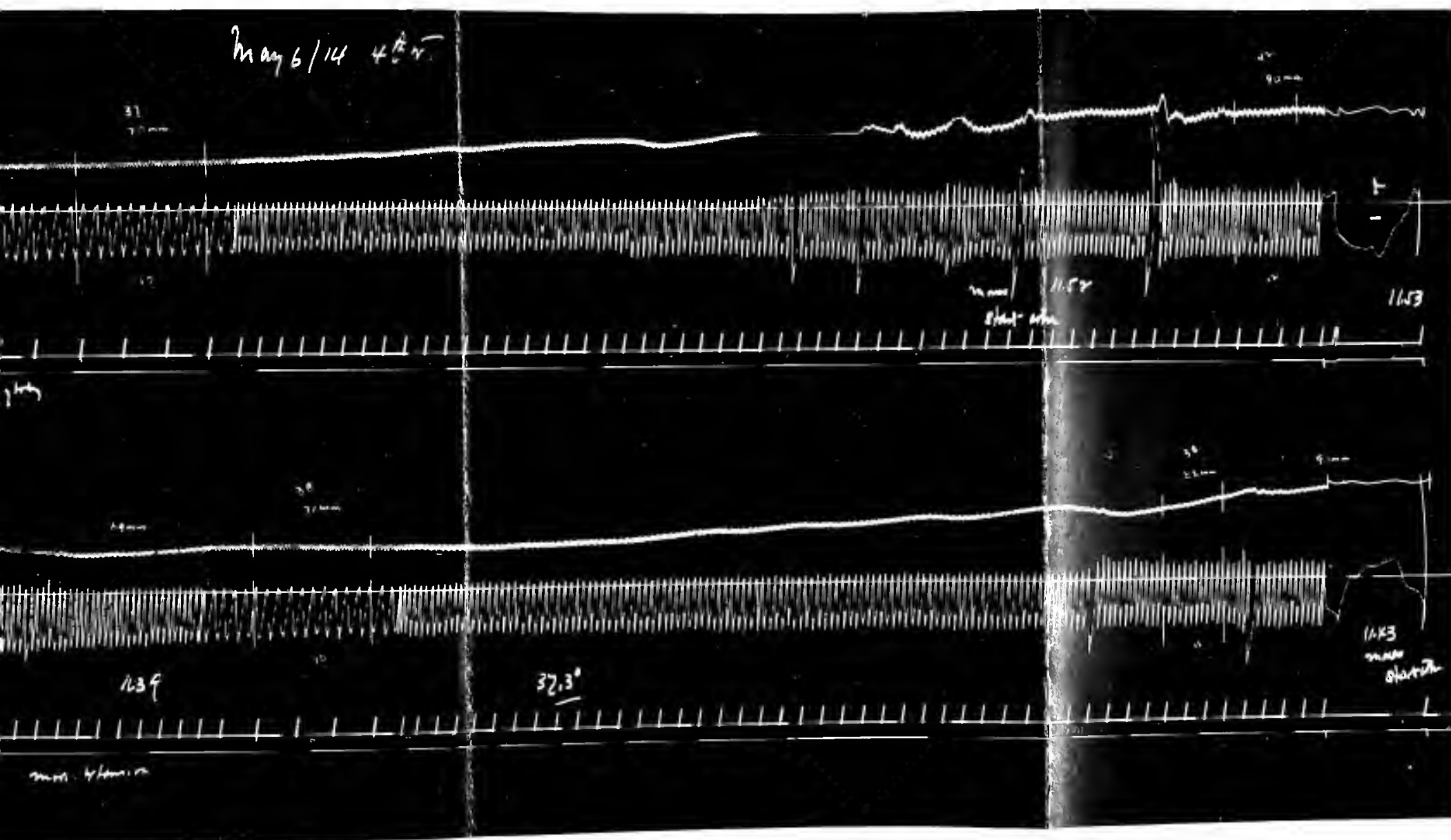


Fig. 5

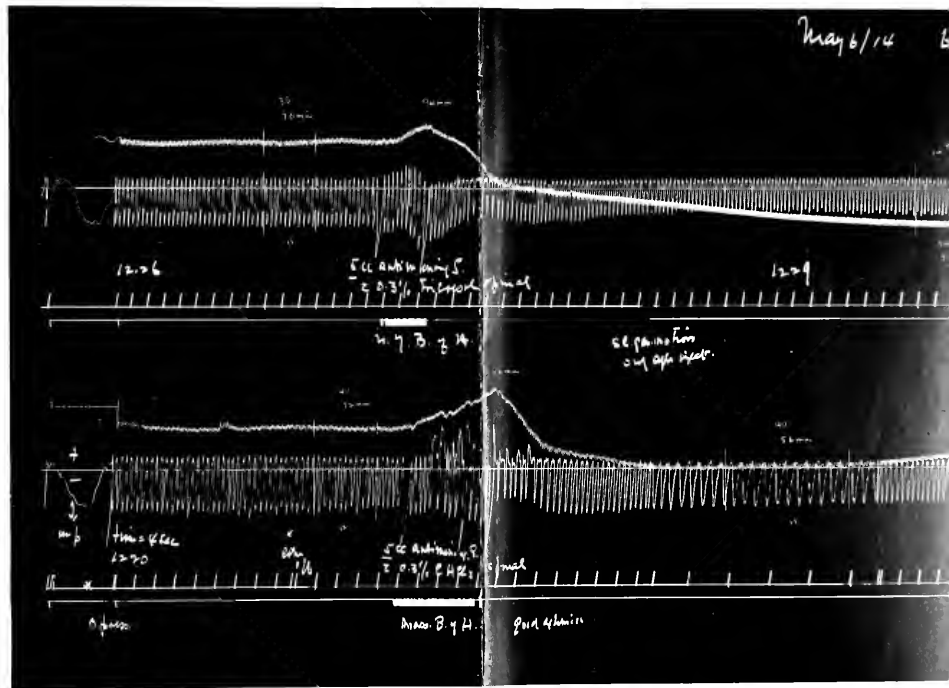
Ampl. Intra-spinal Junctions of Seta 1



PLATE 6.

For same animal as in figure 5, later in the experiment, after the monkey had already received 18 c.c. of 0.3 per cent. chloroform serum and 6 c.c. of 0.3 per cent. trieresol serum (3 c.c. doses usually at 7 minute intervals).

The records the effect exerted by 5 c.c. of chloroform serum injected intraspinaly. Only a moderate effect on the blood pressure and respiration was produced. Seven minutes later 5 c.c. of 0.3 per cent. trieresol serum was injected. With trieresol serum the blood pressure drop was considerably less. Recovery was complete in 7 minutes. The respiration was increased slightly, diminished in amplitude after the trieresol injection. This curve, as figure 3, shows well that a low blood pressure after 0.3 per cent. trieresol serum is not dangerous as long as the respiration is not impaired. This monkey received a number of other injections and tolerated in all 21 c.c. of 0.3 per cent. trieresol serum, 25 c.c. of 0.3 per cent. chloroform serum, and 10 c.c. of Ringer solution, and was in excellent condition at the end of the experiment when the animal was killed. Calcium, cold-red with methylene blue, injected intraspinaly in this monkey at the end of the experiment furnished proof on autopsy that all the injections had entered the dural sac.



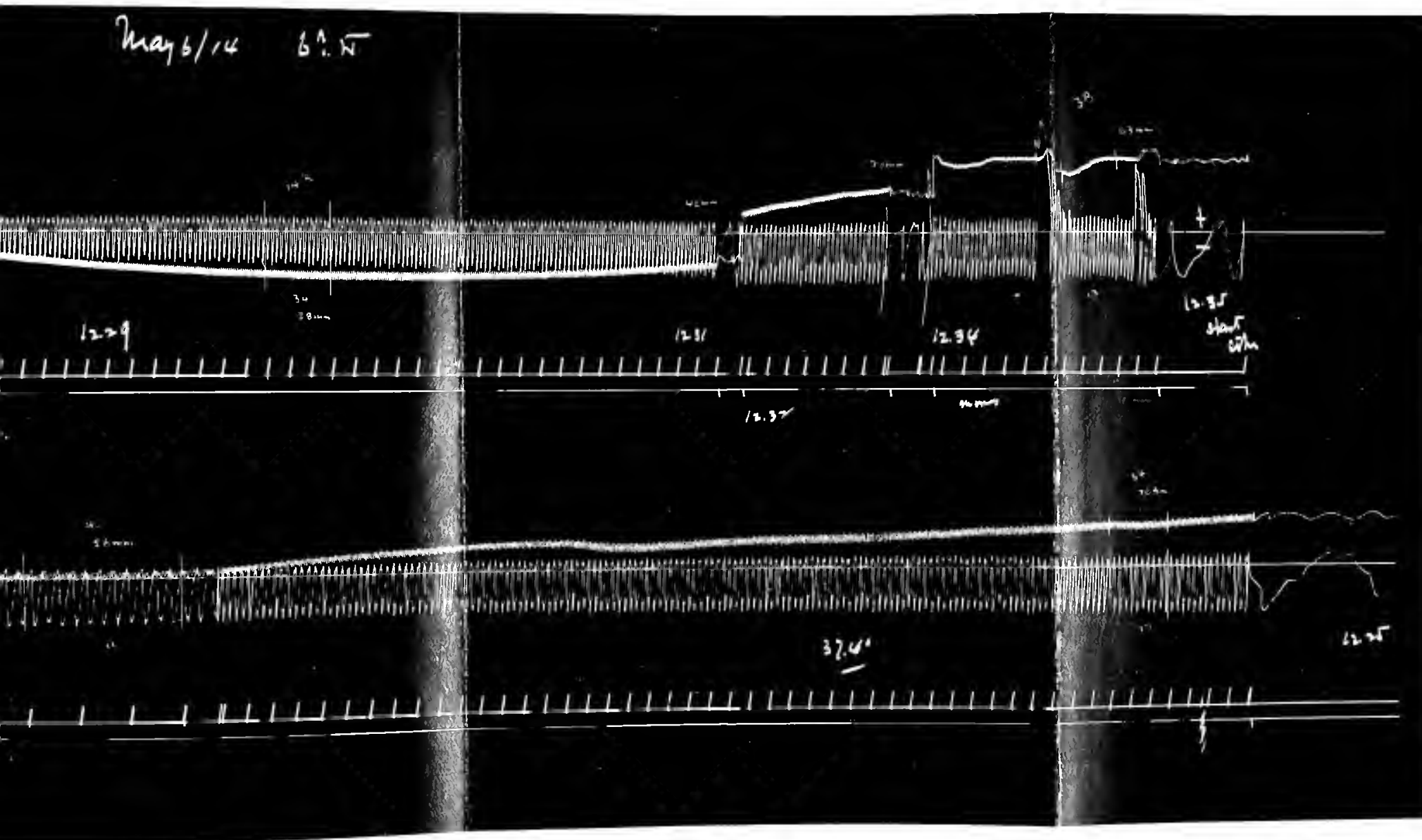


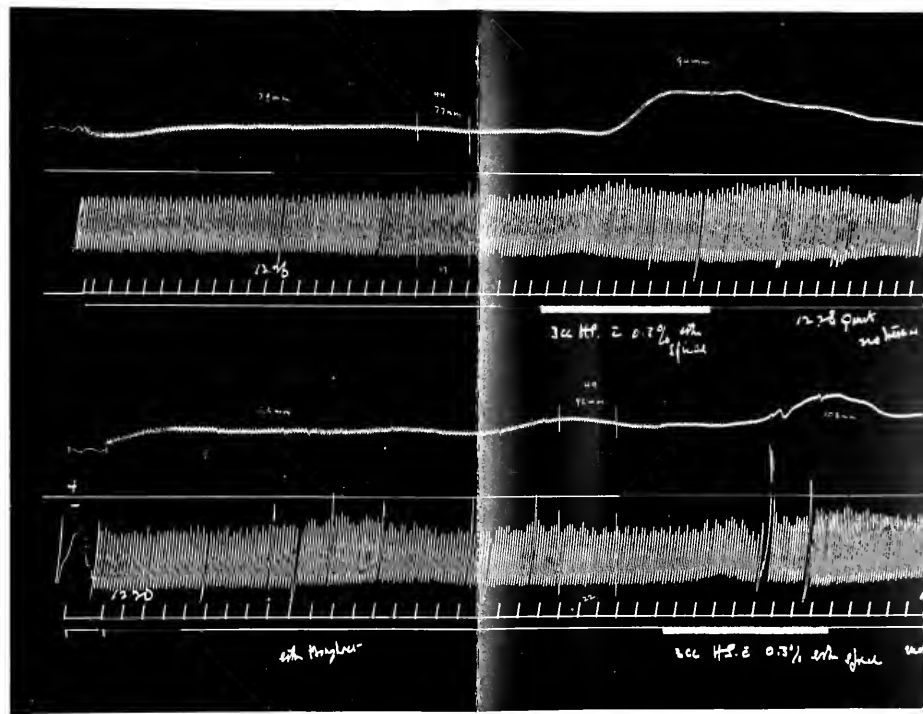
FIG. 6.

Amplitude of Injections of Seta

PLATE 7.

FIG. 7. Monkey 2, ether series; weight 3.575 gm.; vagi intact; normal respiration. This animal had already received 12 c.c. of 0.3 per cent. ether horse serum intraspinally (3 c.c. doses, at 7 minute intervals).

The tracing shows the absence of any decided drop in blood pressure during the first two injections recorded, but the blood pressure tends to remain raised for a period of time. The effect on the respiration is very slight.



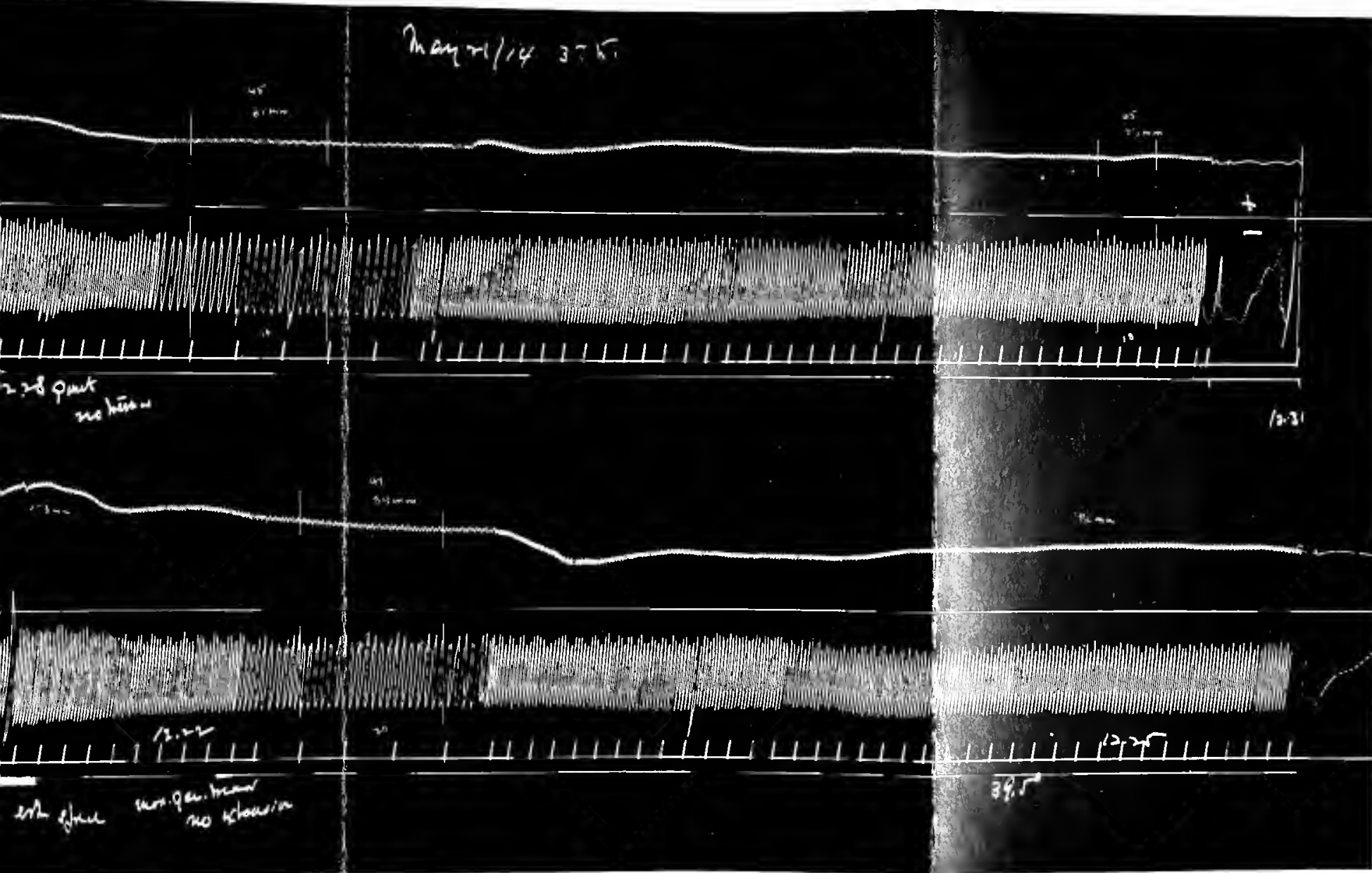


FIG. 7

Amr. Intra-spinal Injections of Sera.

PLATE 8

Monkey, normal serum series; weight 3,205 gm.; vagi intact; no left pneumothorax for 3 days. This animal had been used in experiment on Apr. 28 and had received intraspinally in 3 c.c. dose intervals 15 c.c. of sterile horse serum and 3 c.c. of 0.3 per cent. trichloroethylene, and was in good condition at the end of the experiment, when it was bled and returned to its cage with a left-sided pneumothorax. Four days later, the same animal was in excellent condition and had a graphic record. Previous to the injections noted on figure 1, it had already received in 3 c.c. doses at 7 to 15 minute intervals 15 c.c. of plain horse serum and 6 c.c. of 0.3 per cent. trichloroethylene. The horse serum produced practically no effect on the blood

Table 1 shows the slight effect exerted by a c.c. of sterile plasma on the respiration and blood pressure, but the succeeding intraperitoneal injection of 10 per cent triericed serum caused first a slight short stimulus to the respiration, then a marked slowing and deepening of the respiratory movements in passive expiration. The blood pressure fell promptly after the initial rise, from 70 to 18 mm., and the cardiac rate slowed from 230 to 100. At this point when the condition seemed hopeless, the stopper was removed from the cap of the hypodermic needle which tapped the spinal dura, and fluid dripped away rapidly at first, then more slowly. Within 10 minutes the blood pressure and respiration began to improve rapidly and a few minutes later the animal was in good condition. After 15 minutes the blood pressure was 74 mm., the respirations 60, and the pulse rate 230 per minute. This case shows well the beneficial results of merely reducing the intraspinal pressure in accident cases of this type.

May 1/14 7:15 AM

PLATE 3.

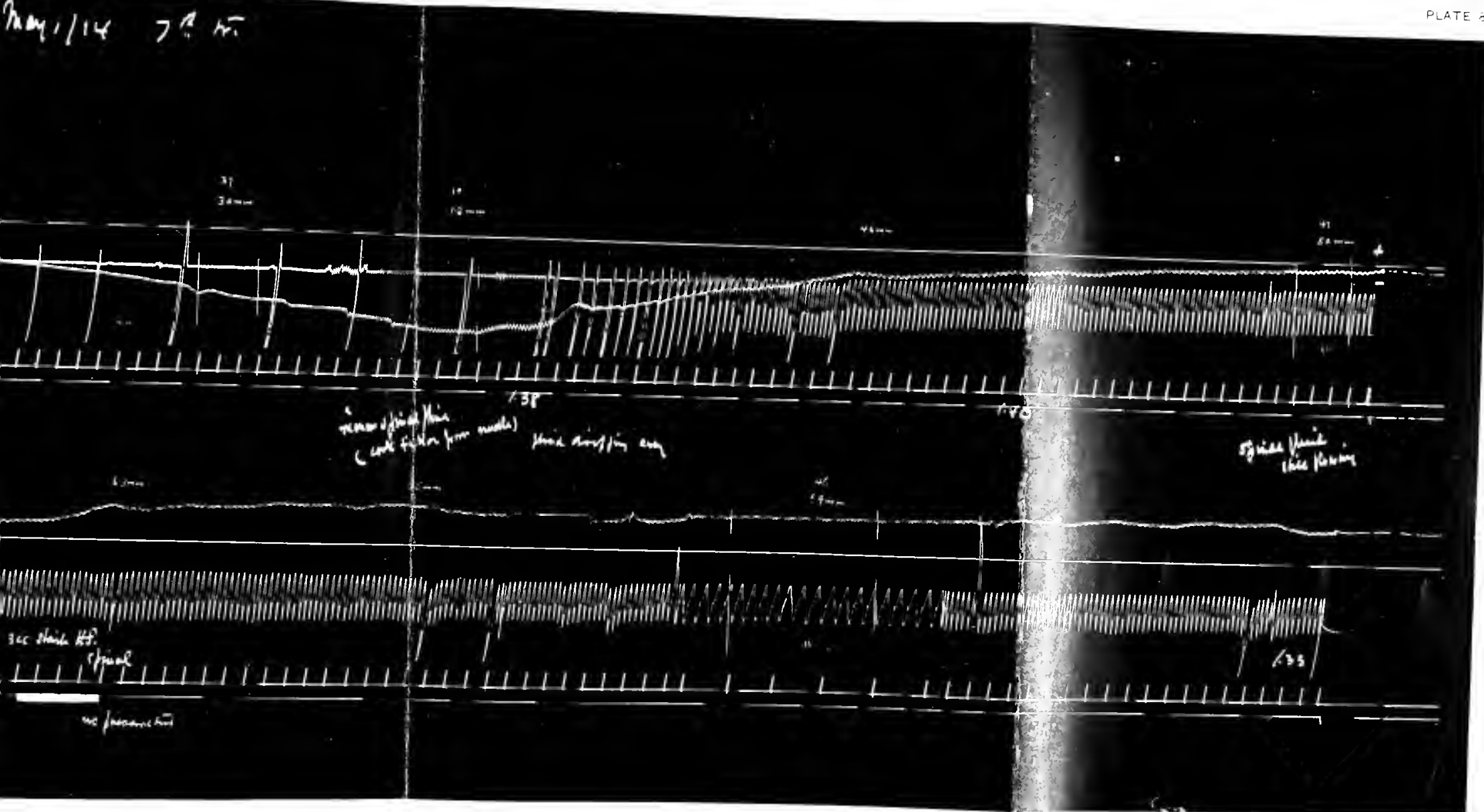


FIG. 8

After Intraspinal Inj. of Serum

Feb 20/1

1635 3.6

110mm 112mm

All Anti-mine down 50.5% Forward 4mm

200000

The first (control) series, weight 5.750 gm., vagi intact, intratracheal throughout, except when marked "off". The first 10 min. shows an exceptionally severe reaction to 5 c.c. of 0.3 per cent. serum injected intranasally for the first time. The respiration showed short stimulation of both rate and amplitude during the injection, and expiration stopped in passive expiration for about 4 minutes (see below). Where the inspiration is discontinued, marked "off" or "m". The large excursions recorded during this time are due to the mechanical relaxations in the absence of the air stream. The blood pressure began to fall before the injection was finished and reached the 42 mm. level, the pulse rate dropping at the same time from 135 to 80 per minute (vagus center stimulation). Recovery of the respiration and blood pressure was prompt and practically normal 10 min. after the injection. This dog tolerated 15 c.c. more of triceps serum and was in excellent condition at the end of the experiment.

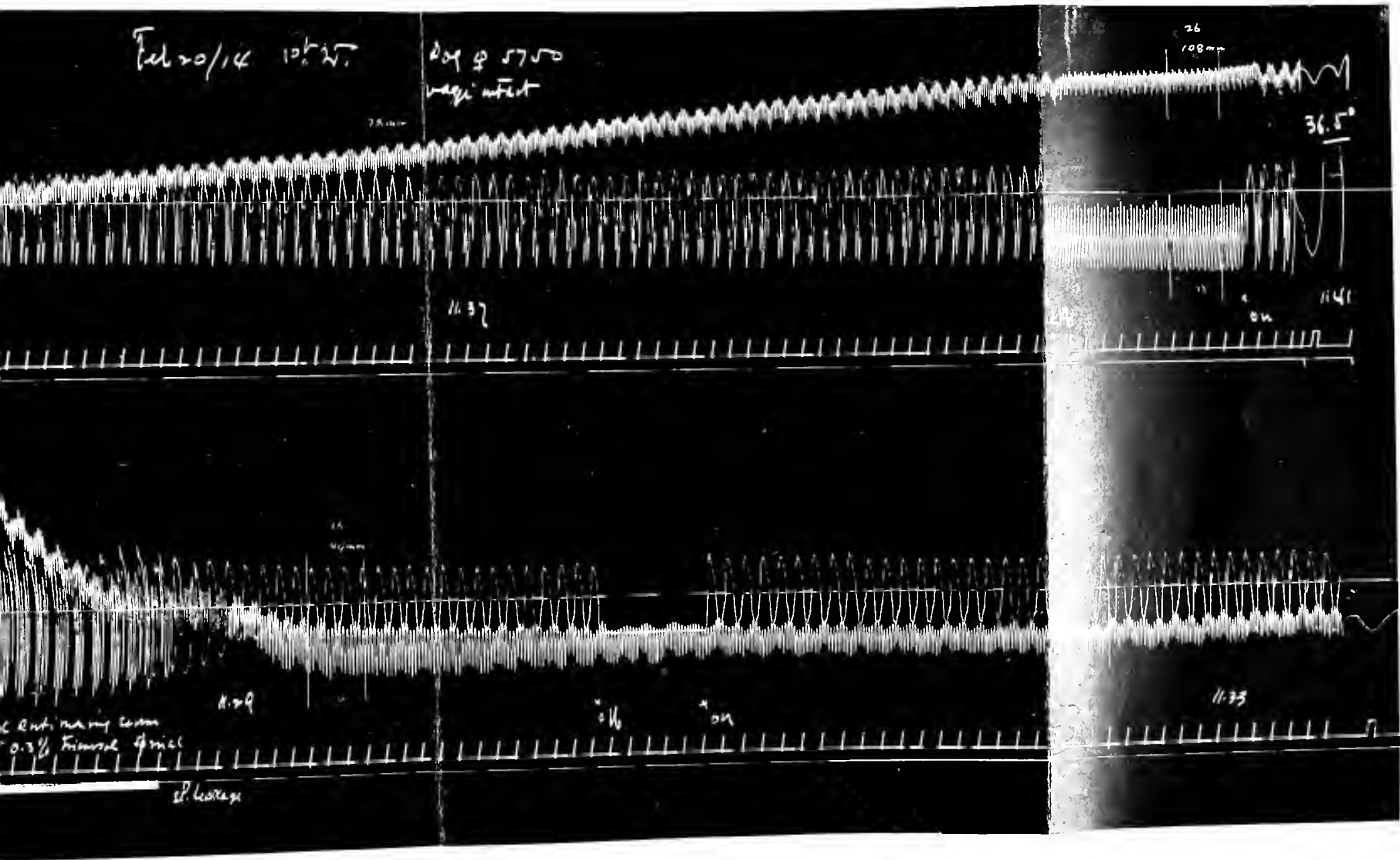
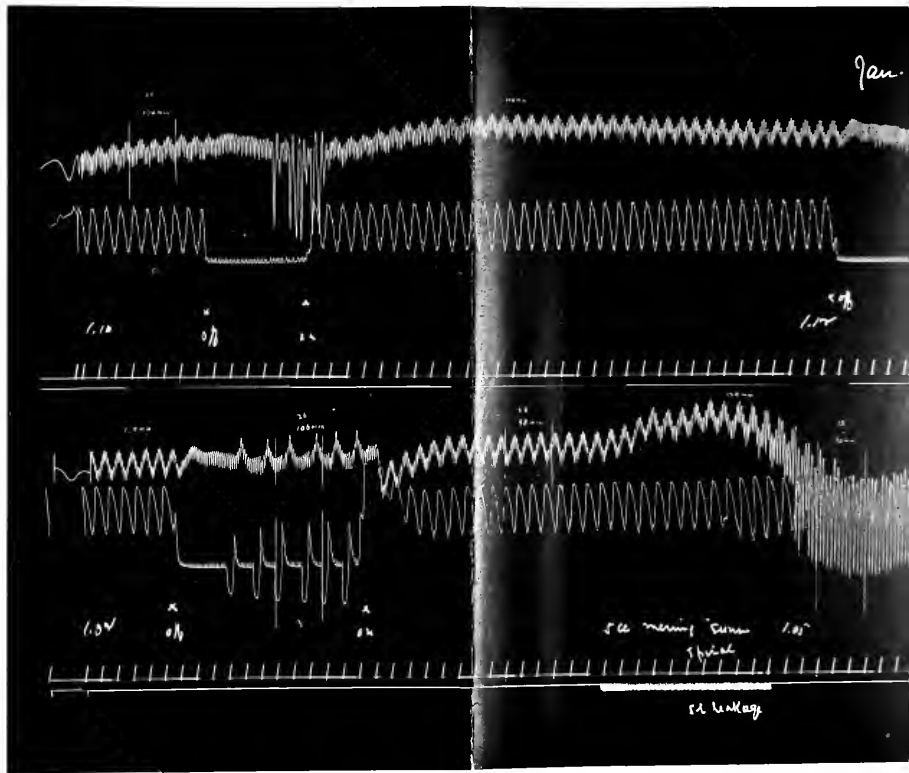


FIG. 9.



On Day 1, tracheal series; weight 4,750 gm.; was intact; intratracheal infusion of air-ether throughout, except when marked "off-on." This dog had already received 30 cc. of 0.3 per cent. trichloral serum before the injection ordered on this tracing. The blood pressure was excellent, 100 mm., but the spontaneous respirations were slow, 15 per minute, and showed inspiratory apnoeas with active expirations (stimulation of inspiration and expiration).

The injection of 5 cc of 0.3 per cent triceol serum now given stimulated respiration but slightly and then caused stoppage of the respiration in passive expiration (inhibition of the respiratory center). This inhibition was a little broken by a series of five respirations each showing a stoppage in the inspiratory position lasting about 4 seconds (stimulation of inspiration); now an inhibition of all respiration again follows, but 7 minutes after the injection spontaneous respirations of the same type as before the injection appear (respiration with inspiratory puffs and active expiration). The blood pressure rose during the injection of the serum from 88 to 120 mm., and then dropped to 60 mm., while the pulse rate fell from 140 to 65 per minute (vagus center stimulation). In spite of this great slowing of the pulse rate the blood pressure began to rise for a time (stimulation of both vagus and vasoconstrictor centers) or some time the blood pressure now escaped occasionally from the vagus stimulation, especially during the group of respirations with long inspiratory pauses. Seven minutes after the injection, respiration, blood pressure, and pulse rate were practically as before the injection. Subsequent injections of 0.3 per cent triceol serum showed the combined stimulation of vagus and vasoconstrictor centers still more markedly.



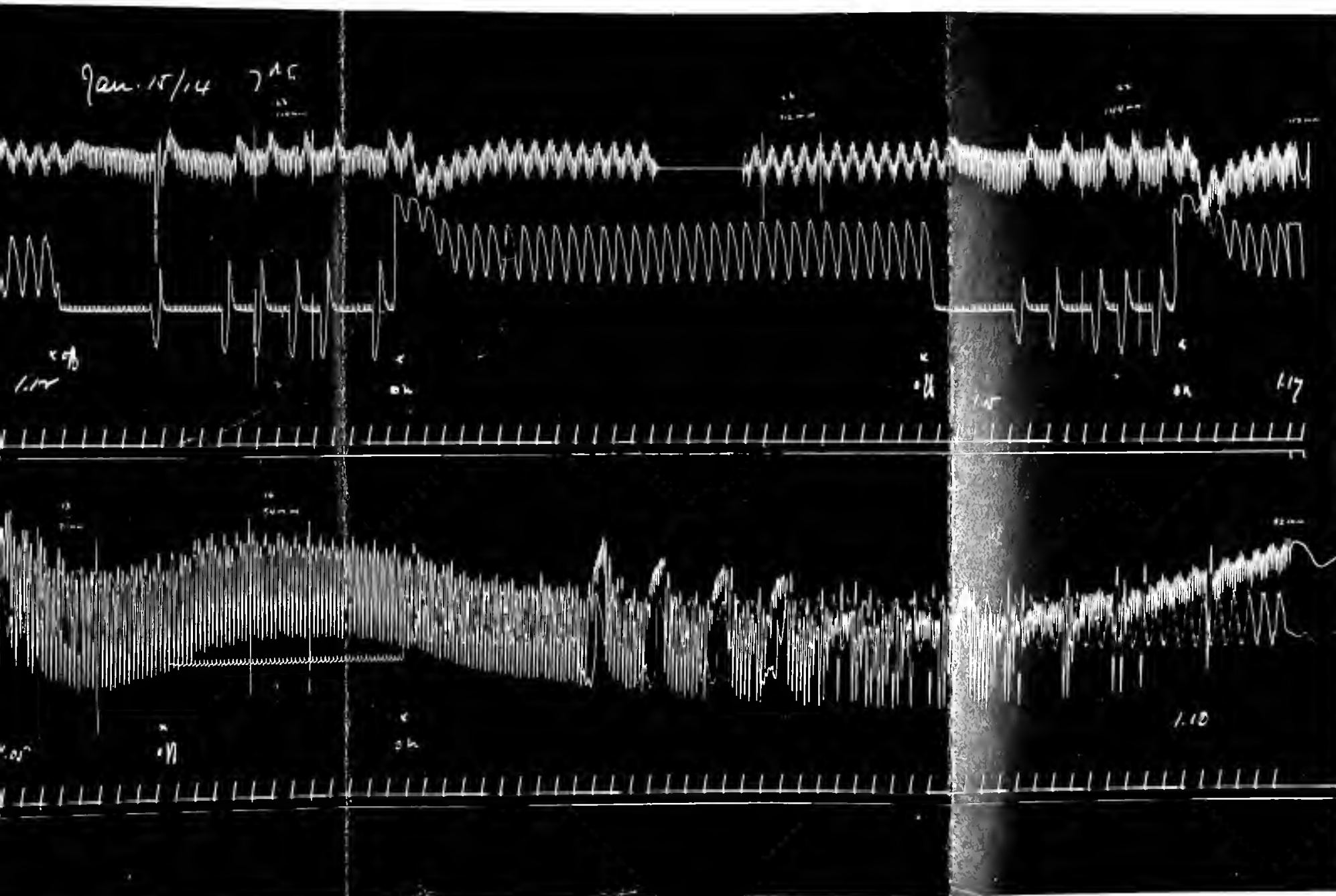


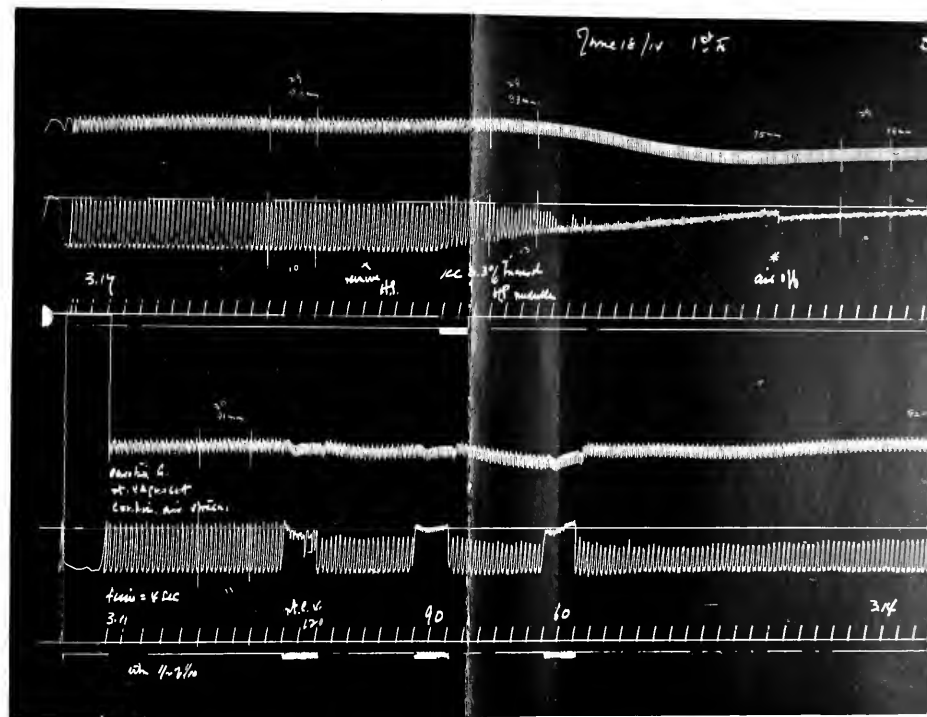
FIG. 10.

(After Intraspinal Injections of Series)

PLATE II

11. Dog 8, medulla series; weight 6840 gm; right vagus cut; left continuous air stream (no remissions); downstroke = inspiration; rt. central stump of the right vagus. The numbers indicate the coil distance in centimeters of a Petzold inductorium driven by two Daniell cells. In this dog the medulla only was exposed.

The lower tracing shows the effect of applying 1 c.c. of sterile plain horse serum to the floor of the fourth ventricle; the blood pressure dropped slightly for short time, but the spontaneous respiration showed no effect. 1 c.c. of 1 per cent tricrosol serum was applied to the medulla after first removing the serum. The respiration first showed an increase of rate accompanied by a decrease in amplitude, which swiftly led to a stoppage of the respiration in expiration. Then the insufflation of air was stopped, but spontaneous respirations began about 1 minute later, at first rapid and of small amplitude, but swiftly the amplitude increased and the rate decreased until the rate was 60 per minute and the amplitude practically normal. It should be noted that the tricrosol serum had not been removed. The blood pressure showed only a slight drop with no change in the pulse rate during the experiment. Stimulation of the central stump of the right vagus caused the same effect upon the respiratory center before and after the application of tricrosol serum. The stimulations caused no strong effect upon the vasomotor centers at any time, although the after effects of the stimuli were slightly more pronounced after the application of the tricrosol serum than before (vasomotor center more irritable after the tricrosol than before). Compare this tracing with figures 9 and 10 when the tricrosol serum was injected intraspinaly.





syringe method in the same animal, but are quite comparable to those noted by me in the monkey (syringe method), which are illustrated in figure 5.

The only experimental discrepancy between Voegtlin's work and mine, where we deal with the same substances in the same strength, is with formalin serum. Voegtlin found that 1:1,000 formalin serum produced the same symptoms as phenol or tricresol injections of 0.25 to 0.5 per cent. strength. The few experiments made by me with 1:1,000 formalin serum yielded a definitely smaller effect on the blood pressure and respiration than 0.3 per cent. tricresol. I have already stated in the body of this paper that the experiments with formalin serum were not continued, because formalin is a powerful coagulant and its action on the therapeutic properties of antisera is unknown.

EDITOR'S NOTE. The employment of curative sera by the intraspinal mode of injection is being constantly extended. Hence the actual dangers inherent and precautions to be observed in carrying out this form of treatment should be clearly understood and appreciated. It is because of the present great practical importance of the subject that this paper has been printed in its entirety, although it exceeds somewhat the length limit prescribed by the *Journal of Experimental Medicine*.

# THE FUNCTION OF THE SPLEEN IN THE EXPERIMENTAL INFECTION OF ALBINO MICE WITH BACILLUS TUBERCULOSIS.

## SECOND PAPER.\*

BY PAUL A. LEWIS, M.D., AND ARTHUR GEORGES MARGOT.

(From the Henry Phipps Institute of the University of Pennsylvania, Philadelphia.)

In a previous paper<sup>1</sup> we presented experiments showing that splenectomized mice are definitely more resistant than intact mice to experimental infection with the tubercle bacillus of bovine type. We have continued our work in the hope of arriving eventually at some adequate explanation of this increase in the resistance of the animal. Our more recent results seem to have some measure of general interest, although they have by no means served to solve the problem under consideration.

Before proceeding to the immediate subject of this paper, some comment on the matter contained in our first paper is necessary. The increase in resistance shown by the splenectomized mice, while unmistakable, has proved to be of a relative character rather than an absolute immunity. In table III of that paper four mice are marked as still living in October, 1913. As regards animals 6 and 13 the table is erroneous. These mice died 120 and 127 days, respectively, after inoculation.

No. 7 died in 306 days, and No. 8 in 314 days. In table IV of our previous paper animals 19 and 20 are marked "still living." These animals died 158 and 164 days after inoculation. All these mice had large numbers of tubercle bacilli in their organs, and there is no reason to doubt that they died as the result of this infection. Moreover, experiments which it is hardly necessary to present in detail have shown that in mice inoculated more than six months after splenectomy the resistance has diminished again to normal or nearly so.

In these animals, when infected for some time with the tubercle bacillus, we have usually found an accessory or perhaps, more properly, an hyperplastic accessory spleen, 1 or 2 mm. in diameter. We have considered it possible that this splenic tissue might be responsible for the loss of resistance occurring thus

\* Received for publication, October 31, 1914.

<sup>1</sup> Lewis, P. A., and Margot, A. G., The Function of the Spleen in Experimental Infection of Albino Mice with *Bacillus tuberculosis*, *Jour. Exper. Med.*, 1914, xix, 187.

spontaneously after some months. With this in mind we have reopened a number of uninoculated mice several months after splenectomy. In these animals we have been unable to find gross evidence of splenic tissue, and it seems probable that these small organs only become evident when rendered hyperplastic by the infection. What influence such subvisible splenic tissue may have on the physiological condition of the animal is problematical.

In a recent paper Murphy and Ellis<sup>2</sup> report experiments in confirmation of our observation of an increase in resistance after splenectomy. Studying mainly the changes in the lymphocytes of the blood in splenectomized and intact animals, with and without exposure to the X-ray, Murphy and Ellis are led to the belief that splenectomy increases resistance by causing an increase in lymphocytes. We have no data bearing on this interesting conclusion.

For the purpose of the present paper, therefore, we use as a point of departure the fact that mice after the removal of the spleen develop in the course of several weeks a well marked although transient increase in resistance to infection with *Bacillus tuberculosis*.

The present series of experiments were made to find out whether the function of the spleen could be wholly or partly replaced by feeding fresh sheep and mouse spleen to splenectomized mice infected with the tubercle bacillus.

In the present experiments the culture used, Bovine C, is one of those previously employed. The methods of splenectomy and infection are those outlined in our previous paper. It should be stated that the usual food of our mice is a mixture of stale bread and oats soaked in water. The animals have been kept in museum jars allowing six by eight inches of floor space. No more than six mice are kept in one jar. The jars are plentifully supplied with fresh wood shavings. The animals are changed at least three times a week to freshly scalded jars. In hot weather or when fresh

<sup>2</sup> Murphy, Jas. B., and Ellis, A. W. M., Experiments on the Rôle of Lymphoid Tissue in the Resistance to Experimental Tuberculosis in Mice, *Jour. Exper. Med.*, 1914, xx, 397.

The tables of Murphy and Ellis show only the average duration of life, and are not particularly convincing. Dr. Murphy has informed us that some of the splenectomized animals lived much longer than any of the controls. It will be noted that in all our tables certain animals are recorded which lived but a few days. These seem to occur without relation to the nature of the experiment. If they are included they greatly disturb the averages except when the groups are very large. In the absence of any definite basis for excluding such animals from consideration, we feel that it is best for the present to discard the average and present the length of life of each individual.

meat has been fed in the jars this change has been made daily. The following tables (experiments I and II) present our more recent results.

#### EXPERIMENT I.

##### *Intraperitoneal Infection.*

Group.	Mice.	Amount of culture.	Subsequent treatment.	Days lived.
I	Intact	1 mg.	None	18, 20, 24, 29.
II	Intact	1 mg.	Fed	16, 20, 29, 29.
III	Splenectomized	1 mg.	None	3, 40, 58, 59.
IV	Splenectomized	1 mg.	Fed	19, 28, 30, 34.
V	Intact	5 mg.	None	17, 20, 20, 26.
VI	Intact	5 mg.	Fed	20, 20, 28, 29.
VII	Splenectomized	5 mg.	None	40, 40, 46, 48.
VIII	Splenectomized	5 mg.	Fed	2, 2, 28, 28.

In this experiment the groups marked under "subsequent treatment" as "fed" received sheep spleen daily. The fresh spleen cut in small pieces was given to them early in the day before they had had other food and was left for several hours so that the animals ate their fill. The reaction of the animals to this method of feeding will be commented on at length in later paragraphs.

#### EXPERIMENT II.

##### *Intraperitoneal Infection.*

Mice.	Amount of culture.	Subsequent treatment.	Days lived.
Intact. . . . .	1 mg.	None	28, 29, 30, 31, 32, 33, 37, 38.
Splenectomized .	1 mg.	Fed fresh sheep muscle	37, 38, 43, 47, 50, 54, 79, 87.
Splenectomized .	1 mg.	Fed sheep spleen	4, 30, 32, 37, 37, 37, 38, 41.
Splenectomized .	1 mg.	Fed mouse spleen	4, 30, 31, 32, 32, 32, 33, 37.

In this experiment the group which was fed with muscle received all that they would eat each day before being offered other food. Those fed with sheep and mouse spleen received an unmeasured amount, but a much smaller one than that available in experiment I. Before receiving other food each mouse in these groups was placed in a separate jar and given a bit of spleen roughly equivalent to one fourth of the usual mouse spleen. The pieces did not exceed the bulk of a small pea.

These experiments have produced additional evidence that splenectomy increases the resistance of mice to infection with the tubercle bacillus. The extreme prolongation of life noted in the experiments reported in the previous paper has not occurred in the present series, but the differences developed in experiment I between the time of survival of groups III and VII and their respective control groups I and V are none the less striking.



It is evident also from the results of experiment I, on comparing group I with II and group V with VI, that the feeding of fresh sheep spleen to intact mice has no influence on the course of their infection. Groups IV and VIII, on the other hand, show that feeding fresh sheep spleen to splenectomized mice leads to the loss of the resistance afforded by the removal of the spleen. Experiment II done at another time with another lot of animals confirms this result and shows further that the feeding of fresh muscle does not affect the consequence of splenectomy.

The feeding experiments were undertaken with the idea that if the resistance could thus be lowered it would be convincing evidence that the spleen was playing a functional part in relation to the infection, and it might be concluded from these experiments that some function affecting the progress of the disease was removed with the spleen and restored with the feeding. There are, however, certain facts in regard to the feeding experiments which lead us to refrain, for the present at least, from regarding the results as decisive. These factors may be discussed briefly under two heads.

1. The gross anatomical features of the disease in the splenectomized mouse fed with spleen differ from those found in the intact mouse. In the intact mice which die in less than thirty days one seldom encounters striking gross lesions in the lungs. Occasionally there are definite gray nodules from one to three millimeters in diameter. Early there may be no visible changes or there may be minute scattered gray points. In the mice which live longer than thirty days, the large nodules are found more frequently, although irregularly. There has seemed to be somewhat more exudation of this type in the splenectomized mice than in the intact mice which have lived longest, but we have been inclined to attribute this to the greater length of life, and to assume that the factors influencing exudation were not grossly changed by splenectomy. The most regular occurrence of large nodules in the lung has, however, been in those splenectomized animals which have been fed with spleen. The significance of this is not clear, and the observation is recorded merely as suggesting that feeding with spleen, while it lowers resistance, does not restore an entirely normal reaction.

2. When normal intact mice are fed daily with fresh spleen, they

eat with apparent relish and maintain their condition perfectly. The intact mice which were fed with spleen after inoculation with tubercle bacilli also ate with relish as long as they could be expected to retain desire for food.

The splenectomized mice when fed with spleen after inoculation took the first one or two feedings freely. After this time it was noted that they seemed not to care for it, and when they did eat of it they were apt to be sick. The illness took no very distinctive character. The animal would go to the corner of the jar and sit huddled up, with rough fur. This usually passed off in a few hours. On the following day, sometimes on the following two days, they would refuse to eat the spleen, although hungry for other food. After two or three days they would again eat spleen, show evidence of illness, and refuse it again on succeeding days. Several mice died in the course of a few hours after eating spleen. Some small hemorrhages in the serous membranes were noted in these animals, but as they were advanced with their infection the cause of death was not clear.

We have fed spleen to uninoculated, splenectomized mice. Of six mice so fed, two died within a few hours after eating spleen. These also showed a hemorrhagic condition of the serous membranes of the peritoneal cavity. The remaining mice showed the signs of illness with a distaste for the food which has been described, but lived and remained in good condition for upwards of two months, when the feeding was stopped. This indicates that there is an irregular, but more or less severe reaction to the feeding of fresh spleen in the case of splenectomized mice.

In view of the fact that uninfected splenectomized mice show some evidence of poisoning when fed with fresh spleen, a final opinion as to the reason for the loss of resistance as developed in the experiments reported in the above tables can hardly be drawn at present. It may be that a true function of the spleen controlling the reaction to the tubercle bacillus is removed by splenectomy and restored by feeding spleen. But it is not impossible that the shortening of life as manifested in the case of the splenectomized, infected, spleen-fed animals is due to some additional poison not directly related to the factors influencing the increased resistance after splen-

ectomy. It is apparently the rule that the resistance of normal animals to infection with the tubercle bacillus is lowered by the continued administration of any poison that we are so far familiar with.

In the hope of getting further light on the reaction of the splenectomized animal to ingested spleen, we performed an experiment with dogs.

Two dogs were splenectomized and allowed to recover from the operation. They were kept for about two weeks on a mixed diet. Then with a normal dog for comparison the feeding was begun. The normal dog was fed entirely for over one month with fresh sheep spleen. He maintained his appetite and condition perfectly. One of the splenectomized dogs gave no reaction to spleen at any time. He was fed in alternate periods of several days with fresh chopped beef and with fresh ground sheep spleen.

The second splenectomized dog was fed from May 27 to June 4 with sheep spleen. On the 3d, 4th, 5th, and 6th days of this period, about twenty minutes after feeding, the animal had what appeared to be a slight chill. He lay down, the hair was roughened, and he had a general shivering tremor. The temperature remained normal. The tremor passed off in a few minutes but the animal remained quiet and indifferent to call for an hour or two. On the seventh day the animal became quiet but did not have the tremor. On the eighth day he had a slight tremor.

From June 5 to June 12 the dog was fed on fresh ground beef. He ate well and remained in good condition.

From June 13 to June 23 he was again fed sheep spleen. The first two days of the period he ate with apparent relish and showed no reaction. On the third day of the period he showed the same symptoms as those described during the first period. On the fourth day he ate, but showed no symptoms. On the fifth day he refused the food at first, but finally ate it and reacted as previously. On the sixth day he again ate with apparent distaste, but completed the meal very soon. He became very quiet and much depressed. For a time he seemed likely to vomit. On the following days of the period he refused to eat at first, but during the twenty-four hours consumed about half the quantity given. At the end of the period he had grown thin and was definitely out of condition.

From June 24 to 29 he was again fed fresh beef with the addition of boiled spleen, ate freely, and condition was rapidly regained. From June 27 to July 9 he was again fed sheep spleen alone, but showed no reaction at any time. He ate freely and maintained good condition.

While the result of this experiment is not entirely convincing, it increases the evidence drawn from the experiments with mice to the effect that fresh spleen is somewhat toxic for splenectomized animals.

## SUMMARY.

The resistance to an infection with the tubercle bacillus which can be given to mice by the removal of the spleen is lost when fresh spleen, either of mouse or sheep, is added to the diet. The logical conclusion that splenic function in its relation to the specific infection is restored by feeding spleen cannot, however, be drawn; because, in the first place, the character of the disease in the splenectomized spleen-fed animals differs somewhat from that in intact animals; and, secondly, because fresh spleen when fed to splenectomized animals apparently gives rise to an acute intoxication.

This intoxication occurs quite regularly in mice. It is manifested in the infected animals and in those not inoculated. It has been seen in one of two splenectomized dogs experimented upon. Up to the present it can hardly be definitely characterized, but it seems to be best marked out by the specific anorexia associated with it.

# CONCERNING SURVIVAL AND VIRULENCE OF THE MICROORGANISM CULTIVATED FROM POLIOMYELITIC TISSUES.\*

BY SIMON FLEXNER, M.D., HIDEYO NOGUCHI, M.D., AND  
HAROLD L. AMOSS, M.D.

(From the Laboratories of The Rockefeller Institute for Medical Research.)

## PLATES 12 TO 17.

In previous reports<sup>1</sup> we described the cultural, morphological, and pathogenic properties of the minute microorganism cultivated from the nervous organs of human and experimental cases of poliomyelitis. We propose in the present paper to describe a strain of that microorganism which survived in a pathogenic state in cultures for a period of thirteen months.

The culture mentioned was obtained originally on November 14, 1912, from a monkey which had succumbed to an inoculation of the M A strain of the poliomyelitic virus,<sup>2</sup> and of which subcultures had proved pathogenic for monkeys in the third, seventh, and twentieth generations.<sup>3</sup> Two tubes of the culture in the ascitic fluid-agar-kidney medium in the second generation had been placed aside, one at room temperature, the other in the thermostat at 37° C., where they remained unmolested until December 20, 1913; that is, for something more than thirteen months. They were now transplanted into the solid medium, and from both sources growths of the minute microorganism were obtained. Hence the cultures survived a period of thirteen months under both conditions. Upon retesting for virulence six months later, that is, eighteen months after isolation, subcultures of this strain proved pathogenic.

\* Received for publication, November 1, 1914.

<sup>1</sup> Flexner, S., and Noguchi, H., *Jour. Am. Med. Assn.*, 1913, lx, 362; *Berl. klin. Wchnschr.*, 1913, l, 1693; *Jour. Exper. Med.*, 1913, xviii, 461.

<sup>2</sup> Flexner, S., and Lewis, P. A., *Jour. Am. Med. Assn.*, 1909, liii, 1639.

<sup>3</sup> Flexner and Noguchi, *Jour. Exper. Med.*, *loc. cit.*

## MASS CULTURES IN FLUID MEDIUM.

From the solid subcultures fluid cultures were prepared by a spécial method to be described. The microörganism multiplies even more readily in a fluid medium consisting of a fragment of rabbit kidney and ascitic fluid overlaid with paraffin oil than in the solid medium. As has been previously explained, an initial culture has never been secured in the solid medium.<sup>4</sup> However, the growth in ascitic fluid is not abundant and hence it is not adapted to the preparation of cultures in mass.

To obviate this difficulty, as well as to eliminate when desired the ascitic fluid, Noguchi employed the double tube method which he devised to secure fluid cultures of spirochætæ.<sup>5</sup> In this method the tube carrying the solid medium, consisting of the kidney fragment, ascitic fluid, and agar, is superimposed upon a tube containing a kidney fragment and ascitic fluid alone or with bouillon, so as to bring the lower end of the agar medium in contact with the surface of the liquid by means of a narrow connecting tube. Thus prepared, the minute microörganism continues to multiply at the point of contact of the two media so that the growth passes into the liquid from which it settles to the bottom of the tube.

This method yields a richer growth than the ordinary ascitic fluid culture and is also suitable for obtaining cultures nearly devoid of ascitic fluid. But, on the other hand, the minute microörganisms begin quickly to degenerate in the medium when the bouillon is predominant. There is probably no actual multiplication of microörganisms, but merely an accumulation.

On this account it is necessary to retain the ascitic fluid. And in order to secure a more abundant growth still the method was modified as follows: Erlenmeyer or Florence flasks of approximately 100 cubic centimeter capacity are charged first with the solid medium. The fragment of kidney having been introduced into the flask, there is placed upon it about 0.5 of a cubic centimeter of a culture of the microörganism, after which fifteen cubic centimeters of a mixture of equal parts of ascitic fluid and 1 per cent. nutrient agar at 40° C.

<sup>4</sup> Flexner and Noguchi, *Jour. Exper. Med.*, *loc. cit.*

<sup>5</sup> Noguchi, *Jour. Exper. Med.*, 1912, xvi, 211.

are carefully poured over the kidney, covering it with a solid layer one centimeter deep. After congelation fifty cubic centimeters of an equal mixture of sterile ascitic fluid and bouillon are introduced, and then a quantity of sterile paraffin oil sufficient to yield a layer of about one centimeter in height (figure 11). The incubation is conducted at 37° C.

The microörganism multiplies throughout the solid medium and, reaching the surface, grows into and within the ascitic fluid bouillon. The large surface of contact facilitates growth, so that at the expiration of about three days the fluid has become highly turbid from the contained microörganisms which have also begun to form a sediment on the surface of the agar medium. Viewed under the microscope the microörganisms are aggregated into small groups chiefly, although short chains are also present, and no degeneration whatever is detectable at this stage of development (figure 1).

#### ACTIVITY OF POLIOMYELITIC VIRUS.

The object of this communication is to record the fact that the microörganism isolated from poliomyelitic tissues may possess specific pathogenic properties after having been cultivated artificially for a period of a year or more, and after an almost indefinite degree of dilution of the original nervous tissues from which it was derived.

It will be recalled that the original cultures are prepared by inserting a fragment of the uncomminuted brain into the ascitic fluid-kidney medium contained in a deep test-tube.<sup>6</sup> When growth takes place, approximately 0.2 of a cubic centimeter is removed with a sterile pipette and transferred to a second deep tube of the medium in which it becomes diffused. The average content of the deep tubes is fifteen cubic centimeters, so that the fluid transferred into the second tube undergoes a seventy-five-fold dilution. From the second generation of the cultures so obtained successive fluid or solid cultures are prepared by a similar transfer of the medium.

The culture with which this paper deals in the second generation was in the solid medium, and from this the third generation, also

<sup>6</sup> Flexner and Noguchi, *Jour. Exper. Med.*, *loc. cit.*

in solid medium, was prepared. From the third generation the combined solid and fluid cultures were prepared. Since the tubes carrying the third generation were of the same capacity as those of the second, the dilution of the materials carried over from the first generation was now approximately 1:5,625. The next, or fourth generation was made in the mixed medium so that the inoculated culture was retained by the congealed agar-ascitic fluid, except as it might diffuse into the overlying ascitic fluid-bouillon. If we assume that the diffusion was uniform, which of course it was not, the dilution brought about by the seventy-five cubic centimeters of combined solid and fluid media would now be approximately 1:843,750. From now on each successive culture yields such a rapidly diminishing content of any material originally carried over as soon to approach the infinite.

The minimal effective dose of the poliomyelitic virus represented by filtrates prepared from emulsions of nervous organs of 2.5 to 5 per cent. strength is about 0.001 of a cubic centimeter.<sup>7</sup> However, only occasional strains of the virus are as active as this. At an early period after the adaptation of the M A virus to monkeys it possessed this extreme degree of virulence. At the period at which the cultures were prepared the activity of the M A filtrate had diminished at least 100 times. In other words, the minimal effective dose had risen to 0.1 to 0.2 of a cubic centimeter. Since the quantity of brain tissue employed in the cultivations is about 5 per cent. of the volume of fluid (one gram of brain to fifteen cubic centimeters of ascitic fluid), the calculated potency of the fluid, irrespective of any increase of the virus and assuming that all diffused into the surrounding medium, would have been 0.2 of a cubic centimeter, which is the average quantity transferred to the tubes composing the second generation of cultures.

This consideration is affected by the period of survival of the poliomyelitic virus at 37° C. Incomplete observations only are directly available covering that point. Flexner and Lewis<sup>8</sup> observed that a Berkefeld filtrate mixed with rabbit serum and bouillon and inoculated at 37° C. was active at the expiration of ten days,

<sup>7</sup> Flexner, *Lancet*, 1912, ii, 1271; *Science*, 1912, xxxvi, 685.

<sup>8</sup> Flexner and Lewis, *Jour. Am. Med. Assn.*, 1910, liv, 45.



and Levaditi<sup>9</sup> noted a similar mixture to be still active after fifteen days. Hence it is doubtful whether the limits have been reached in the tests.

On the other hand, the limits have been reached in another series of tests which, in themselves, have an especial significance for us. Conceiving a possible interaction or symbiosis between nerve cells and the microbe of poliomyelitis, Levaditi<sup>10</sup> cultivated in normal monkey plasma *in vitro* fragments of the intervertebral ganglia of paralyzed monkeys. Successive transplantations having been made, the survival and increase of the virus were determined by inoculation. In one experiment the virus was still present at the fourth passage on the twenty-first day. In a second experiment it was present in the second passage on the thirteenth day, but not later. In the third and final experiment the first and second passages at seven and thirteen days respectively were effective, while later passages were devoid of infective power. The inoculation of the plasma alone in which the fragments grew was ineffective. Levaditi regards this failure as explained by the fact that the microbe multiplies only in association with living cells of tissue and not in the surrounding fluid. The point of these experiments that concerns us here relates to the period of survival of the virus in an effective state, the maximum being twenty-one days.

This result supports the observation reported by Flexner and Noguchi;<sup>11</sup> namely, that when culture tubes, set up with brain tissue, are tested for virulence as early as the second generation in the ascitic fluid medium, which is at the twentieth day or later after the original preparation, they are only exceptionally effective and then only when growth of the minute microorganism has taken place. The conclusions are therefore: (a) only exceptional strains of the culture are pathogenic for monkeys; (b) the virus diffusing into the fluid from the brain tissue soon deteriorates or undergoes dilution beyond the effective dose.

<sup>9</sup> Levaditi, C., *Presse méd.*, 1910, xviii, 44.

<sup>10</sup> Levaditi, *Compt. rend. Soc. de biol.*, 1913, lxxiv, 1179; 1913, lxxv, 202.

<sup>11</sup> Flexner and Noguchi, *Jour. Exper. Med.*, *loc. cit.*

## EXPERIMENTAL.

The fourth and subsequent generations of the culture, used for inoculation purposes, were prepared in the fluid medium in the manner for obtaining the abundant or mass growth of the microörganism. The strain was kept pure and ready for transfer to the fluid medium by implantation from time to time into the solid medium. The fluid is, of course, far more subject to contamination during manipulation than the solid culture; and contamination is more readily detected in the latter.

The inoculation of monkeys was performed not with a single dose but with several doses of the fluid culture. The reason was two-fold. Earlier studies had shown that only exceptional cultures of the microörganism are capable of infecting monkeys; and although the strain of microörganism now available had at the time of original isolation been effective, the retention of pathogenic power over the long period since its removal from the body was regarded as improbable. On the other hand, Flexner and Lewis<sup>12</sup> had observed that in process of immunization of monkeys with subcutaneous injections of the ordinary virus there sometimes supervened not increased resistance but paralysis. Hence successive inoculation offered two possible and opposite sets of effects: (1) infection might be induced, and (2) immunity might be secured. In the series of experiments to be described immediately the former was accomplished.

The culture in the second generation which survived thirteen months was subcultured into solid medium until June 2, or eighteen months after its isolation. It was in the ninth generation. The culture was now transplanted to the combined solid and fluid medium in flasks and subcultured in that medium until June 27, when it was employed for the inoculation of monkeys. At this time it had passed through more than twelve generations.

*Experiment 1.*—*Macacus rhesus*. June 27. 1 c.c. of the fluid culture was injected intraspinally by lumbar puncture. June 29. Lumbar puncture yielded turbid fluid containing red and white corpuscles and a few small masses of the injected microörganism staining indistinctly. No growth was obtained in cultures from the spinal fluid. July 3. No symptoms. 2 c.c. of the next generation of

<sup>12</sup> Flexner and Lewis, *Jour. Am. Med. Assn.*, 1910, liv, 1780; 1910, lv, 662.

fluid culture injected intraspinally. July 9. No symptoms. 4 c.c. of fluid culture injected intraspinally. July 20. No symptoms. A new series of fluid cultures had been prepared with a mixture of the stock solid cultures ranging from the fourth (made on Jan. 15) to the tenth (made on July 1), and of the fluid so prepared 4 c.c. were injected intraspinally. July 25. Legs spastic; animal ataxic. July 26. All four extremities paralyzed; animal prostrate; died the same day.

*Autopsy.*—The membranes about the spinal cord were edematous. The pial vessels at the base of the brain were congested. Section of the spinal cord at several levels showed gross lesions resembling those of poliomyelitis. A block of brain tissue was employed for cultivation tests. Portions of the spinal cord and medulla were put aside in 50 per cent. sterile glycerin.

*Histology.*—Sections from the spinal cord, medulla, and intervertebral ganglia were studied. The lesions are those of severe poliomyelitis. The meningeal and vascular infiltrations are pronounced (figures 2 and 3); the anterior gray matter of the spinal cord shows extensive necrosis and neurophagocytosis of nerve cells (figure 4); the medulla is the seat of focal infiltration of the vessels and nervous tissue (figure 5); the intervertebral ganglia exhibit nodular and diffuse cellular infiltration and necrosis of nerve cells. The accumulations in the interstitial tissue consist chiefly of mononuclear cells, while the necrotic nerve cells are invaded by polymorphonuclear neurophages (figures 6 and 7). A part of the ganglia show extensive diffuse necrosis of nerve cells and cellular infiltrations.

*Experiment 2.—Macacus rhesus.* The cultures inoculated were obtained from the same source as the preceding. June 27. 2 c.c. of the fluid culture injected into the peritoneal cavity. July 3. No symptoms. 4 c.c. of the culture injected into the peritoneal cavity. July 20. No symptoms. 10 c.c. of the mixed culture injected into the peritoneal cavity. July 30. No symptoms. 20 c.c. of the culture employed on July 20 injected into the peritoneal cavity. Aug. 3. Animal moves about slowly. Aug. 4. Prostrate. Aug. 5. Dead.

*Autopsy.*—The organs generally and the peritoneal cavity appeared normal. The spinal cord showed at several levels lesions resembling those of poliomyelitis. A block of brain tissue was removed for cultivation tests. Portions of the spinal cord and medulla were put aside in 50 per cent. glycerin.

*Histology.*—Sections from the spinal cord, medulla, and intervertebral ganglia were studied. All show typical poliomyelitic lesions. The meningeal infiltration is less than in experiment 1. The perivascular infiltrations in the medulla are especially pronounced (figure 8), and the sheaths of the larger vascular branches are edematous.

*Experiment 3.—Control.* When the first monkey of this series responded on July 26 with symptoms of poliomyelitis, a control inoculation was decided upon. This was carried out on July 30 at the same time that the last injection was given to experimental monkey 2 and with the culture employed for that inoculation. 4 c.c. of the fluid were injected intraspinally into a *Macacus rhesus*. No symptoms developed.

*Experiment 4.*—Two additional tests were made with the glycerinated specimens put aside from experiments 1 and 2. Emulsions of the spinal cord and medulla preserved in glycerin were injected intracerebrally into two *Macacus*

*rhesus* monkeys. The emulsion from monkey 2 was inoculated on Aug. 11; symptoms appeared on Aug. 15, progressed, and the animal was etherized on Aug. 24. The lesions in the spinal cord, medulla, and intervertebral ganglia were typical of poliomyelitis. The emulsion from monkey 1 was inoculated on Sept. 14; symptoms appeared on Sept. 24, progressed, and the animal was etherized on Sept. 26. The lesions in the nervous tissues are, in this instance, typical also. Some of the intervertebral ganglia show especially severe lesions in which, besides diffuse infiltration, the nerve cells in wide areas are hyaline and degenerated and the small periganglionic cells proliferated (figures 9 and 10).

The symptoms, lesions, and infectiousness of the glycerinated tissues, as represented by data given, are further indicative of the power possessed by the cultures to set up in monkeys experimental poliomyelitis.

Moreover, the microorganisms inoculated were recovered in the cultures prepared with the brain tissue of monkeys 1 and 2. The recovery was accomplished not readily but with difficulty, just as in the case of the brain tissue from human subjects or from monkeys infected with the ordinary virus. This point is significant; for it appears that the acquisition of parasitic properties by the microorganism unfits it for ready multiplication in artificial culture media.

#### DISCUSSION.

The data presented in the foregoing pages are believed to bear essentially on the question of the etiological relationship to epidemic poliomyelitis of the minute microorganism cultivated from poliomyelitic tissues.

Since the publication of our first full paper on this microorganism, several members of the staff of the Institute have made attempts to isolate it from nervous tissues and with a degree of success which attended the previous efforts. In other words, the cultivation was accomplished in some, but not in all instances, and success is determined by various circumstances; by the sample of ascitic fluid, the degree of anaerobiosis, the original sterility of the rabbit kidney, as well as by some other factor or factors at present unknown and uncontrollable. It is this last circumstance that makes the result uncertain; but it is one, fortunately, which experience tends to eliminate. Practice in the work of cultivation leads to greater assurance of success; and hence those who undertake the cultivation

should use many samples of ascitic fluid, employ different means of obtaining anaerobic conditions, and should not be easily discouraged by failure. In view of the difficulties mentioned, the fact should be emphasized that while this general method for the cultivation of refractory microorganisms devised by Noguchi has been employed extensively at the Institute, in no instance has a microorganism resembling that isolated from poliomyelitic tissues been obtained from any other source than that described.

It is significant and illuminating in this connection to find that when poliomyelitis has been incited in monkeys by means of cultures, the recovery of the microorganism is made with difficulty. This fact suggests the conclusion that the reacquisition of parasitic properties by the microorganism contained in cultures unfits it for ready multiplication in an artificial medium, which is, however, well suited to the growth of the same microorganism undergoing saprophytic development.

Perhaps this peculiarity of behavior may account for the wide fluctuation in pathogenic action shown by different cultures of the microorganism, since exceptional strains only are effective. Pathogenic power does not depend wholly on the culture generation and thus on distance from the parent stem. A non-virulent culture is already ineffective in the second generation, from which fact the side conclusion can be drawn that activity is not, probably, dependent on mechanical admixture of an invisible virus derived from the brain tissue employed in the cultivation.

The experiments reported in this paper not only confirm the earlier successful results, but extend them in a way to strengthen the evidence in favor of the etiological relationship of the minute microorganism to epidemic poliomyelitis. An experiment now to be described adds support from another side.

It had been established that the only certain way in which immunization to poliomyelitis may be experimentally secured in monkeys is by passing them through an attack of the disease. Inoculations of the poliomyelitic virus which are ineffective afford no protection to a subsequent effective inoculation. By carrying forward with great care successive injections of a virus, immunity may be established in some, but not in all instances. But the successive injections may

unexpectedly and for unknown reasons suddenly produce paralysis just when an immunity might have been inferred.<sup>13</sup>

And the same series of events have been observed with the cultivated microorganism. Successive injections of a culture wholly devoid of virulence yield neither infection nor immunity, and the monkeys so treated possess no neutralizing principles in the blood for the virus, and develop paralysis promptly after inoculation of an active sample. The following experiment illustrates this point.

*Experiment 5.—Macacus rhesus.* Successive subcutaneous injections of fluid cultures contained in the liquid portion of Noguchi's double tubes were given over a period of several months. The condition of the animal remained good throughout the treatment. Apr. 23. Withdrew blood in order to obtain serum for a neutralization test with ordinary virus. 1 c.c. of the serum failed to neutralize either 0.1 or 0.3 c.c. of the usual filtrate. June 12. Injected 0.2 c.c. of a filtrate of the ordinary virus intracerebrally. June 22. Excitable; ataxic; tremor. June 24. Arms paralyzed, legs weak. June 25. Dead.

Microscopical examination of the central nervous organs showed typical lesions of experimental poliomyelitis.

In contradistinction to the results of this experiment, which shows the lack of immunizing power of inert cultures of the minute microorganism, are experiments 1, 2, and 3, which establish the important fact that a culture may be ineffective at the first, and effective at a subsequent injection, although the material employed for the final injection may prove inactive in a fresh monkey. The deduction from the experiments seems to be that in the course of successive inoculation, under certain circumstances, the resistance of the monkeys is gradually suppressed, and that this undermining takes place more quickly when the cultures are introduced into the cerebrospinal membranes than when injected into the peritoneal cavity.

#### SUMMARY.

The minute microorganism cultivated from poliomyelitic tissues survives and maintains its pathogenicity in cultures for more than one year.

Upon inoculation into monkeys poliomyelitis may fail to appear upon the first injection and yet follow from the effects of successive injections of the culture.

<sup>13</sup> Flexner and Lewis, *Jour. Am. Med. Assn.*, 1910, liv, 1780; 1910, lv, 662; *Jour. Exper. Med.*, 1910, xii, 227. Flexner, *Jour. Am. Med. Assn.*, 1910, lv, 1105.

Inoculations of cultures into monkeys which fail to produce paralysis may fail also to induce resistance or immunity. In this respect the action of the cultures resembles that of the virus as contained in infected nervous tissues.

The lesions occurring in the spinal cord, medulla, and intervertebral ganglia of the monkeys which respond to the several inoculations of the cultures are identical with those present in the nervous organs of the animals responding to injection of the ordinary virus.

Glycerinated nervous tissues derived from the monkeys responding to several injections of the cultures transmit experimental poliomyelitis to monkeys upon intracerebral inoculation.

The microorganism inoculated may be recovered in cultures from the monkeys which develop poliomyelitis; but cultivation from the brain tissue is attended with the usual difficulties surrounding the obtaining of the initial growth.

The microorganism cultivated from poliomyelitic tissues is adapted with difficulty to saprophytic conditions of multiplication, but once adapted growth readily takes place upon suitable media. When, however, as a result of inoculation into monkeys, the parasitic propensities of the microorganism are restored, it again displays the marked fastidiousness to artificial conditions of multiplication present at the original isolation.

The experiments reported in this paper afford additional strong evidence in support of the view already expressed, that this microorganism bears an etiological relationship to epidemic poliomyelitis in the human subject and to experimental poliomyelitis in the monkey.

#### EXPLANATION OF PLATES.

##### PLATE 12.

FIG. 1. Sediment showing the minute microorganisms after three days' growth in mixed ascitic fluid and bouillon in a flask employed for mass cultivation. Giemsa stain.  $\times 1,000$ .

FIG. 2. Spinal cord showing meningeal cellular infiltration extending into the anterior median fissure. Experiment 1.  $\times 100$ .

##### PLATE 13.

FIG. 3. Spinal cord showing perivascular cellular infiltration in the meninges and white matter. Experiment 1.  $\times 85$ .

FIG. 4. Anterior horn of spinal cord showing necrosis and neurophagocytosis of ganglion cells. Experiment 1.  $\times 170$ .

PLATE 14.

FIG. 5. Medulla oblongata showing focal cellular invasion of the gray matter and perivascular cellular infiltration. Experiment 1.  $\times 110$ .

FIG. 6. Intervertebral ganglion. Interstitial cellular infiltration. Experiment 1.  $\times 250$ .

PLATE 15.

FIG. 7. Intervertebral ganglion. Two necrotic ganglion cells invaded with neurophagocytes. Experiment 1.  $\times 200$ .

FIG. 8. Medulla oblongata. Perivascular cellular infiltration in floor of fourth ventricle. Experiment 2.  $\times 106$ .

PLATE 16.

FIG. 9. Intervertebral ganglion. Diffuse cellular invasion of interstitial tissue and necrotic ganglion cells. Experiment 4.  $\times 150$ .

FIG. 10. Same as figure 9, showing several ganglion cells in which the periganglionic cells have proliferated and neurophagocytes have invaded degenerated cells.  $\times 250$ .

PLATE 17.

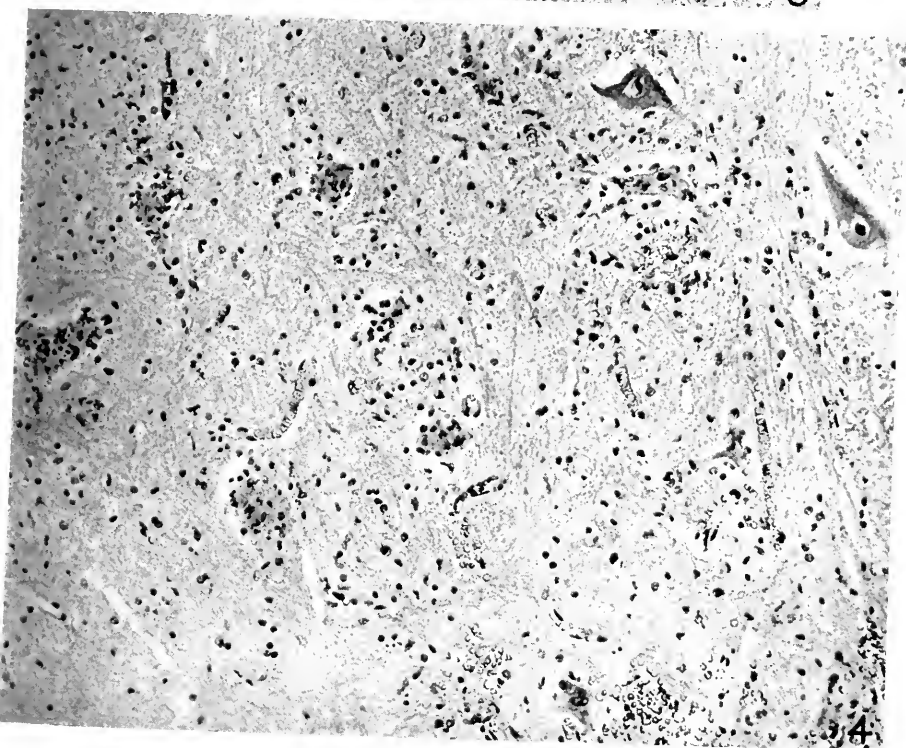
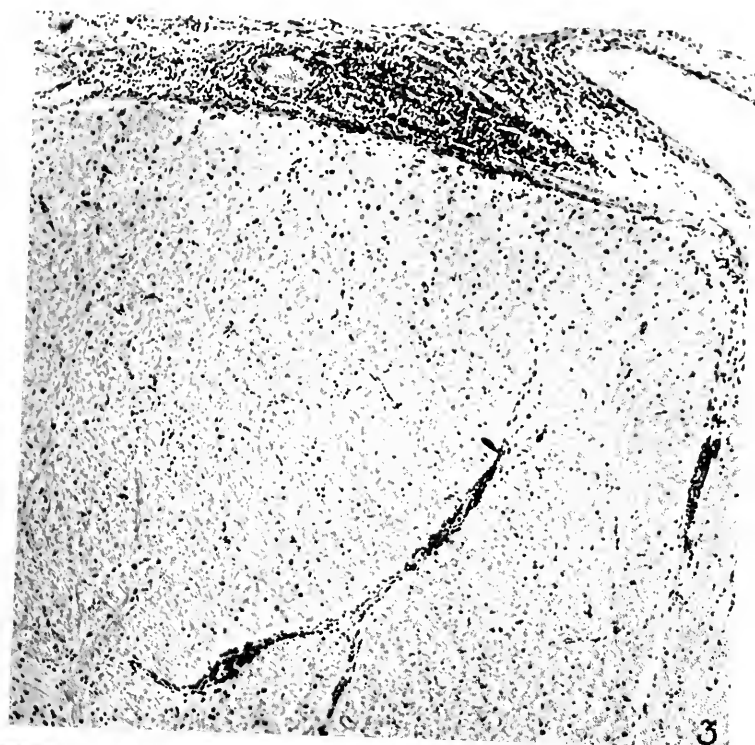
FIG. 11. Mass culture. 1, uninoculated control flask; 2, inoculated flask; a, layer of paraffin oil; b, layer of ascitic fluid-bouillon; c, layer of ascitic fluid and agar containing fragment of rabbit kidney.



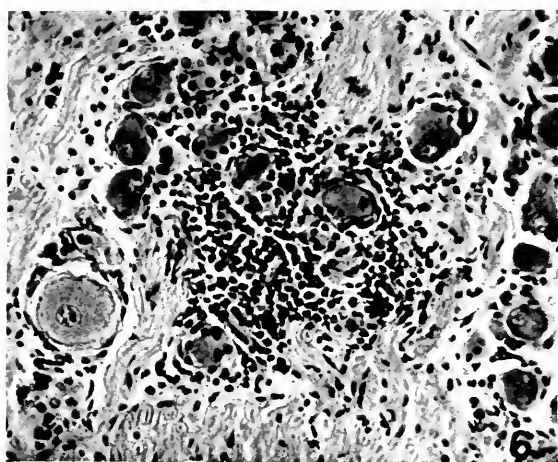
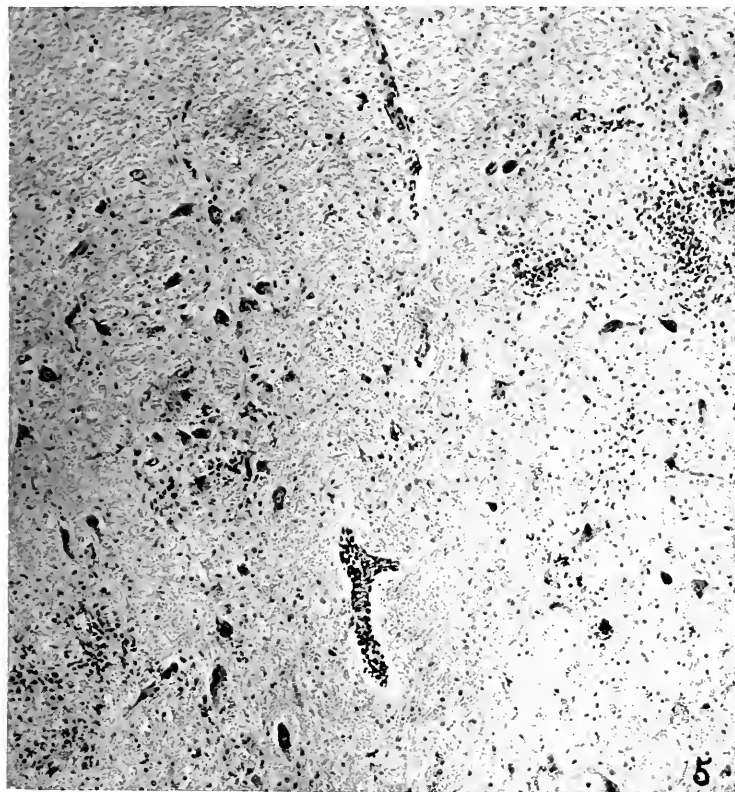


(Flexner, Noguchi, and Amoss: Survival and Virulence of Poliomyelitic Microorganism.)



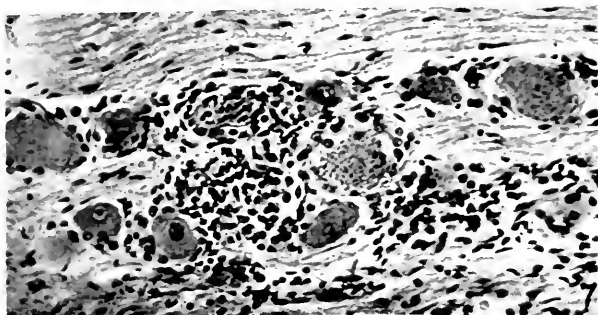






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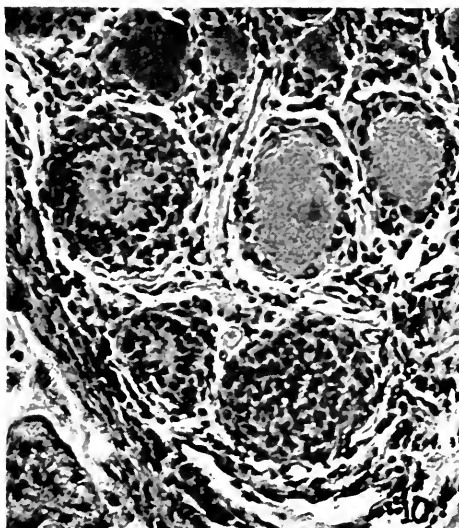
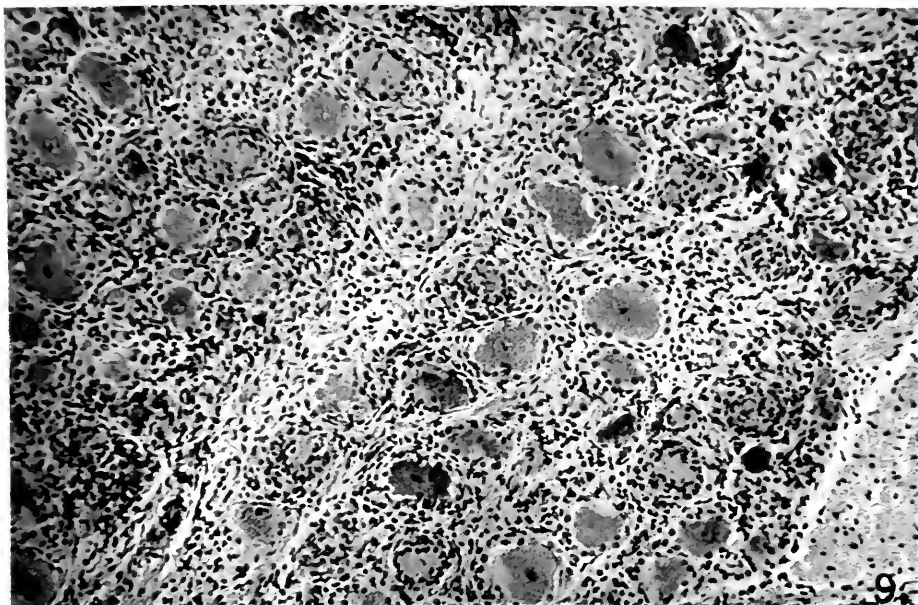




(Flexner, Noguchi, and Amoss: Survival and Virulence of Poliomyelitic Microorganism.)

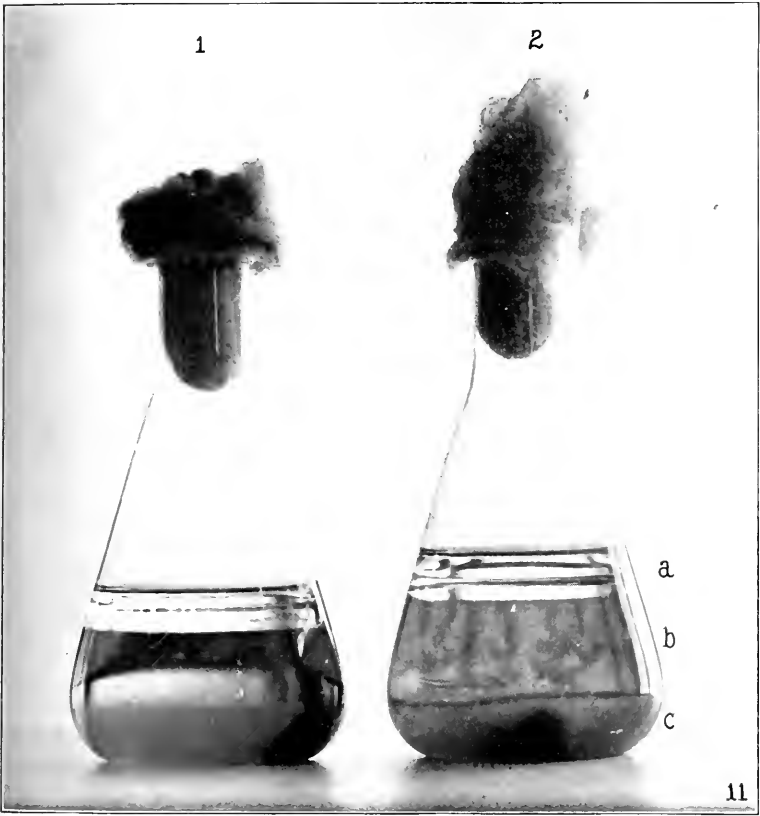






(Flexner, Noguchi, and Amoss: Survival and Virulence of Poliomyelitic Microorganism.)





(Flexner, Noguchi, and Amoss: Survival and Virulence of Poliomyelitic Microorganism.)



# THE REACTIONS BETWEEN BACTERIA AND ANIMAL TISSUES UNDER CONDITIONS OF ARTIFICIAL CULTIVATION.\*

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PLATES 18 TO 20.

Realizing that in ordinary bacterial culture media as usually employed the conditions are far from ideal, and at best a lifeless inactive medium in contradistinction to the active living medium presented by the human or animal body, I was led, in the fall of 1913, to undertake a series of experiments with tissue cultures according to the now well known method of Harrison as modified by Burrows (1). These actively growing cell cultures were used as media on which to attempt cultivation of various pathogenic organisms, and some interesting and suggestive results were obtained which are reported here as opening up what seems to be a promising vista for more detailed observations along the same lines. In these growing cultures the cells are constantly splitting the protein bodies of the plasma to form amino-acids which even bacteria with no proteolytic enzymes can easily handle, and also here we have the opportunity, hitherto lacking, of observing from day to day, directly under the microscope, the interaction of bacteria and tissue cells in normal homogenous or heterogenous plasma, in plasma from immunized animals, and in artificial media which may be varied at will.

## TECHNIQUE.

In most of the studies here reported chick embryo cultures were employed, especially heart tissue and splenic tissue from seven to fourteen day embryos. The former gives two indications of bacterial action, the rate and extent of cell multiplication and the rate and force of pulsations or the arrest of the same; while in splenic cultures connective tissue growth and leucocytic growth and migra-

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tion may be studied. Bacterial inoculations may be made on solidified cultures at varying intervals after making, into diluted plasma at or before the time of tissue planting, or as controls into plasma without tissue. With each series of cultures sterile controls of normal tissue should always be made, as conditions vary with each planting and results are valueless without comparisons under identical conditions of tissue, plasma, temperature, etc. In all inoculations, except those on solidified cultures twenty-four hours or more after planting, the natural bactericidal action of the plasma must be taken into consideration.

The organisms studied so far have been *Micrococcus aureus* and *Bacterium diphtheriticum*, both pathogenic to chickens and to man, a Hoffman type *Bacterium pseudodiphtheriticum* for comparative purposes, *Bacillus typhosus*, pathogenic to man but not to chickens, *Bacillus coli verus*, usually non-pathogenic and normal to both man and chickens, and *Bacillus prodigiosus*, a non-pathogenic air organism. The diphtheria and typhoid organisms gave the most interesting and characteristic results, but the actions of all organisms used were instructive when comparisons were made. Cultures were made in series and observed daily, and specimens were fixed and stained for permanent preparations at regular intervals. With diphtheria, observations on the action of toxin and antitoxin on tissue cultures were also made. In judging results it must be remembered that one is dealing with very minute fragments of tissue, and to approach normal conditions, bacterial inoculations should be very light. In most series two classes of cultures were made, small cover-glass hanging block preparations with one or two drops of diluted plasma and one to four tissue fragments, and larger cultures on the under surface of lids of glass boxes, the culture containing five to ten drops of diluted plasma and numerous tissue fragments. Where plasma inoculations are made the best plan is to inoculate the diluting fluid, sterile water, or Ringer's saline solution, before adding it to the plasma, and if light inoculations are desired two or more dilution tubes in series should be used, as in plating on agar. A dilution of one part water or saline to two parts plasma gives good results for routine work. In all instances agar slants were made from inoculated plasma tubes to control the inoculations.

## BACILLUS PRODIGIOSUS AND MICROCOCCUS AUREUS.

As the two extremes of action, *Bacillus prodigiosus* and *Micrococcus aureus* may be taken. *Prodigiosus* failed to develop colonies in chicken plasma without tissue, and in most cases also in the presence of tissue. Thiele and Embledon (2) claim that when inoculated from a hypertonic medium *prodigiosus* becomes pathogenic owing to the protective action of the saline preventing early bacteriolysis, and in confirmation of this fact it was found that when *prodigiosus* was grown in hypertonic bouillon (3 per cent. sodium chloride) it would invariably develop a few colonies in tissue cultures. In contrast with *prodigiosus*, *Micrococcus aureus* invariably grew very freely and vigorously under all conditions in such cultures with or without tissue, and with all tissues used. With most tissues, while the *aureus* colonies were abundant throughout the culture, they were larger and more numerous near the tissue fragments and entirely prevented or very much inhibited tissue growth. With splenic cultures, however, and to a less degree with embryonal liver tissue cultures, this was not the case. If inoculations were heavy the colonies clustering around such tissue fragments were fewer and much smaller, while with moderate and light inoculations these tissues were surrounded with clear bacteria-free halos almost coëxtensive with the areas of migration of the lymphatic cell elements. This contrast between heart and splenic tissue action is well shown in figure 1 where fragments of both tissues have been planted in the same infected culture.

## BACILLUS TYPHOSUS AND BACILLUS COLI VERUS.

*Bacillus typhosus* and *Bacillus coli verus*, closely related to each other in morphology and cultural characteristics, showed marked contrasts in tissue cultures, more sharply marked than in ordinary laboratory differential tests for the same. *Bacillus typhosus* never in a single instance in ninety tests showed any development in plasma cultures, with or without tissue, when inoculated from isotonic or from hypertonic media. That this was due to strong bactericidal action of the chicken plasma for *Bacillus typhosus* was shown by the following results: when freshly solidified cultures or six hour old

cultures were inoculated with this organism, by means of a platinum needle dipped in a heavy suspension, no bacterial growth occurred; but if the cultures were first incubated for twenty-four hours and then inoculated there was usually a light spreading growth; and if incubated for forty-eight hours before inoculation there was always a heavy growth, the incubation having destroyed the complement at least, if not the amboceptor, in the plasma which had prevented bacterial development. That the bacteria were actually killed was shown by lack of growth on agar slants from such cultures after incubation. *Typhosus* growth developing on such incubated cultures did not interfere with tissue cell growth or pulsation of heart tissue for at least forty-eight hours, or until the tissue cells were heavily coated with the encroaching bacteria, which had a tendency, in old cultures especially, to cling to the new cells like vines to a decaying tree, giving the picture in stained cultures referred to by the author as "bacterial trees," as seen in figure 2. This same appearance was seen with one other organism, a spore-forming rod of the *subtilis* type developing as an accidental contamination. In one instance only was it possible to overcome the bactericidal action of fresh plasma on *Bacillus typhosus*; here the moist tissue fragments were covered with a heavy suspension of active typhoid organisms several minutes before the plasma was added, thus giving an extremely heavy dose of organisms in proportion to plasma. In contrast with the failure of typhoid bacilli to develop in tissue cultures, *Bacillus coli verus* seems to be entirely uninfluenced by any bactericidal properties of chicken plasma and to grow freely in vigorous colonies generally scattered throughout the plasma and, if the inoculation is heavy, to have a decidedly inhibitory action on tissue cell growth. Growth was also vigorous in clear plasma without tissue fragments. The clear halos seen around splenic fragments with *Micrococcus aureus* were absent with *coli verus*, the colonies developing up to and on the splenic tissue as vigorously as elsewhere.

BACILLUS DIPHTHERITICUM AND BACILLUS PSEUDO-  
DIPHTHERITICUM.

The most interesting results of bacterial infections of tissue cultures were those obtained with *Bacterium diphtheriticum* in contrast



to those with *Bacterium pseudodiphtheriticum*. With heavy inoculations both organisms develop freely, with or without tissue fragments, but when tissue is present by far the most vigorous colonies are seen immediately surrounding the tissue fragments. With diphtheria bacilli in such cases the tissue makes no growth and heart fragments do not pulsate, but with pseudodiphtheria bacilli, even with heavy inoculations, there is some new cell growth, and heart fragments may at times pulsate when laden with colonies. With moderate or light infections, however, there is a far more marked contrast. Pseudodiphtheria bacilli in light inoculations only occasionally develop and then only an individual colony survives here and there through the plasma, this happening more often in the smaller cover-glass preparations than in the box cultures. If inoculated from hypertonic bouillon, however, it usually developed more freely, as did also *prodigiosus*. Diphtheria bacilli in light or moderate inoculations showed a striking and peculiar development, the colonies appearing in numbers clustered around the individual tissue fragments, as in figure 3, and never in the clear plasma away from tissue, or in plasma controls with no tissue. In box cultures containing numerous tissue fragments each fragment resembled a planet with numerous satellite colonies around it. With splenic tissue the colonies clustered around the outer limits of the zone of lymphatic cell migration, leaving a clear zone next to the original tissue which resembled a sun, the center of a microscopic solar system (figure 4). The bacilli, ordinarily destroyed, if not in too great numbers, by the bactericidal action of the plasma, seem to be afforded some protection or stimulation by some cell secretion or product of cell metabolism which enables them to overcome the bactericidal influence of plasma. This fact may explain why clinically diphtheria is a local infection and rarely, if ever, a bacteremia, except in pre-agonal states where the plasma has lost its bactericidal properties. The clear, bacteria-free halo was lacking around a culture of splenic tissue from a seven day chick embryo, but at this time the spleen has little, if any, lymphatic elements and shows a pure connective tissue type of growth. That the failure of bacilli to grow in plasma without tissue was due to a bacteriolysis was shown by comparing the results of cultures made from the same batch of plasma one

and four days after its inoculation with diphtheria organisms, in the former case there being numerous colonies clustered around and through the heart tissue fragments, while in the latter there was only an occasional colony developed. As a general rule, heart cultures infected with diphtheria bacilli pulsated less vigorously and ceased pulsation much earlier than sterile controls in the same series, and when solidified cultures, after incubation, were inoculated pulsation usually ceased within twenty-four hours after the bacterial growth had reached the tissue fragment. With moderately heavy plasma inoculations the tissues at first showed very slight evidence of new growth, as in figure 3, but after twenty-four to forty-eight hours, when the cells seem to have produced antitoxin enough to overcome the toxic action of the colonies, some new cell growth begins to take place. Previous addition of diphtheria antitoxin to the plasma did not influence bacterial growth, but it did prevent deleterious action of the bacilli on the tissue cells. An interesting confirmation of the close relation between true diphtheria bacilli and pseudodiphtheria bacilli was seen when pseudodiphtheria inoculations were made into plasma containing diphtheria toxin. In these cases pseudodiphtheria organisms behaved just as did true diphtheria in ordinary cultures, the colonies developing regularly and always clustering around the tissue fragments.

#### SPLENIC EXTRACT.

To endeavor to determine if the clear halos around splenic cultures seen in *aureus* and *diphtheria* inoculations were due entirely to phagocytosis of bacteria by lymphatic cells, extracts of embryonal splenic pulp were prepared according to the method used by Carrel (3) in his work on growth stimulation, and these extracts, after storage for ten days or more on ice, were dropped on solidifying cultures of tissue with bacteria. In every instance the area covered by the splenic extract remained free, or nearly free, from colonies, while the remainder of the culture was filled with them. Embryonal liver, also rich in lymphatic elements, had a similar action to that of spleen, though not to so marked a degree.

## DIPHTHERIA TOXIN.

In addition to the above experiments with bacterial inoculations a series of experiments was carried out to show the action of diphtheria toxin and antitoxin on cell growth. The toxin was added in varying amounts to the plasma before culturing, the dosage usually being calculated for the amount of plasma, not of tissue which was subject to more variation, in proportion to toxin, from the standard of the L. + dose of toxin employed for a 250 gram guinea pig; 0.5 of a cubic centimeter of plasma, the amount used for one box culture, containing 1/500 or N/500 of the L. + dose. In general, chick tissues seem to have a much higher resistance to toxin than do guinea pigs, for one L. + dose had little or no effect on cultures. Larger doses, however, had an increasing inhibitory action and if sufficiently large the growth and pulsation of heart tissue were prevented, as were growth and migration of lymphatic elements from splenic tissue. Moderately heavy doses, not sufficient to kill tissue, arrested growth for twenty-four to forty-eight hours, after which time some new cells began to develop. Growth of nervous tissue was arrested by decidedly smaller doses than was that of any other tissues. New cells developing from cultures with large doses of toxin were few in number and showed early advanced degeneration, heavy accumulation of fat droplets, blunted processes, and dense inactive or fragmented nuclei (figure 5). Cultures from the same series with the same dose of toxin but with also a corresponding dose of antitoxin showed fairly vigorous growth with many normal or nearly normal cells (figure 6).

## SUMMARY.

This report gives an outline of the results of observations on over 1,100 tissue cultures made during the fall, winter, and spring of 1913 to 1914. The work has been resumed in the fall of 1914 and will be continued along the same and allied lines, confirming the above results with other strains of the same organisms and with other bacteria and bacterial products. Tests should be made with tissue and plasma from other animals, and the known pathogenicity of the organism for the animal and tissue used should always be

borne in mind. We hope to be able to grow on these cultures some of the more strictly parasitic bacteria not developing on ordinary media, as other strict parasites have been grown by other workers, *viz.*, poliomyelitis virus by Levaditi (4), vaccinia by Steinhardt, Israeli, and Lambert (5), and rabies by Moon (6). By comparative studies with various types of cells and various natural and artificial media, clearer ideas as to the exact part of cell plasma in antibody production, by elaborating on the methods of Carrel and Ingebrigtsen (7), Lüdke (8), Przygode (9), and others, should be possible.

After this work was begun a reference was found to the use of some pathogenic bacteria in tissue cultures by Pheiler and Lentz (10), but no publication of the results of these observations has been observed.

The results here reported may be summarized as follows:

*Bactericidal Action of Chicken Plasma*.—On *Bacillus typhosus*, very strong—never grows in plasma alone; on *Bacillus prodigiosus*, very strong—never grows in plasma alone; on *Bacterium pseudodiphtheriticum*, strong—slight growth in cover-glass preparations; on *Bacterium diphtheriticum*, moderately strong; on *Bacillus coli verus*, slight; on *Micrococcus aureus*, very slight or none.

A few pseudodiphtheria bacilli and more diphtheria bacilli survived in plasma stored in the cold for four days. The presence of growing tissue overcomes the bactericidal influence of plasma on diphtheria bacilli and in some instances on pseudodiphtheria bacilli.

*Bacterium diphtheriticum* grows in plasma without tissue only if inoculations are very heavy; and very heavy inoculations of all organisms will probably overcome the bactericidal action of plasma, as it is undoubtedly a quantitative reaction. The bactericidal influence of plasma is overcome by exposure to incubator temperature for twenty-four to forty-eight hours. *Bacterium diphtheriticum* in light or moderate inoculations grows in tissue cultures only in clusters around the tissue fragments, and never in plasma away from tissue. The growth of this organism has a decided inhibitory influence on tissue activity and growth, especially marked with nervous tissue, but this action may be overcome by the addition of antitoxin to the plasma. Cultures inhibited by diphtheria growth

have a tendency to resume growth later, probably due to antitoxin production.

*Bacterium pseudodiphtheriticum* is distinctly less active in tissue cultures than is *Bacterium diphtheriticum* and never develops in plasma without tissue. The presence of diphtheria toxin in tissue cultures causes this organism to behave as does *Bacterium diphtheriticum*. Without toxin it has little or no direct influence on tissue growth except in massive doses.

*Bacillus prodigiosus* fails to develop, as a rule, in tissue cultures except where inoculated from hypertonic media, and then it has no decided influence on tissue growth.

*Micrococcus aureus* grows freely in these cultures with or without tissue, and inhibits tissue growth markedly, except as noted with splenic tissue.

*Bacillus coli verus* always grows freely with or without tissue fragments and is uninfluenced by splenic tissue growth. In heavy inoculations it lessens tissue growth.

*Bacillus typhosus*, except with extremely heavy inoculations, fails absolutely to grow in these cultures with or without plasma, unless the bactericidal action of the plasma has been destroyed by incubation. When this is the case it develops freely with especial affinity for the tissue cells either for support or nourishment. It appears to have no toxic action on the tissue cells. Note the sharp differentiation between typhoid and *coli verus* organisms.

Diphtheria toxin has a quantitatively inhibiting action on all tissue growth and on heart tissue pulsations, the action being greatest on nervous tissue and least on heart tissue growth. Tissues affected by toxin tend to recovery if not killed. Antitoxin counteracts the action of toxin.

Splenic tissue has little or no effect on the growth of *Bacillus coli verus*, but has a decided bactericidal action on *Bacterium diphtheriticum* and *Micrococcus aureus*, probably due to lymphatic cells and cell products, as seen by the area of cell migration coinciding with the bacteria-free area, by the similar action of splenic extract on cultures, and by the failure of such action in cultures of very early splenic tissue showing no lymphatic cells.

## CONCLUSIONS.

We have in tissue cultures *in vitro* a promising addition to our methods of bacteriological study. The reaction of bacteria to tissue cultures would seem to be more or less parallel to the pathogenicity of the organism for the animal supplying the tissue.

This method promises a reliable means of differentiation between some pathogenic and non-pathogenic organisms of the same species. It will probably shed more light on the protective action of tissue cells and cell products against bacterial action, as is seen with splenic cultures.

It will probably explain more clearly the action of pathogenic bacteria and the definite relation they bear to the tissues involved and to the blood plasma, as is seen from the peculiar behavior of *Bacterium diphtheriticum* in tissue cultures.

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## EXPLANATION OF PLATES.

## PLATE 18.

FIG. 1. Twenty-four hour culture from twelve day chick embryo heart and splenic tissue in plasma inoculated with *Micrococcus aureus* before planting. Notice the limited tissue growth and general distribution of bacterial colonies, except in the clear halo around the splenic fragment. Magnified 30 diameters. H = heart fragment. S = splenic fragment. B = bacterial colonies.

FIG. 2. Seven day culture from eleven day chick embryo heart tissue in plasma. This culture was inoculated with *Bacillus typhosus* on the fifth day of incubation and shows new cell growth heavily invested with bacilli ("bacillary trees") with few or no bacilli between the cells. Magnified 220 diameters. H = heart fragment. C = new cell growth. T = bacteria-laden cells.



FIG. 1.

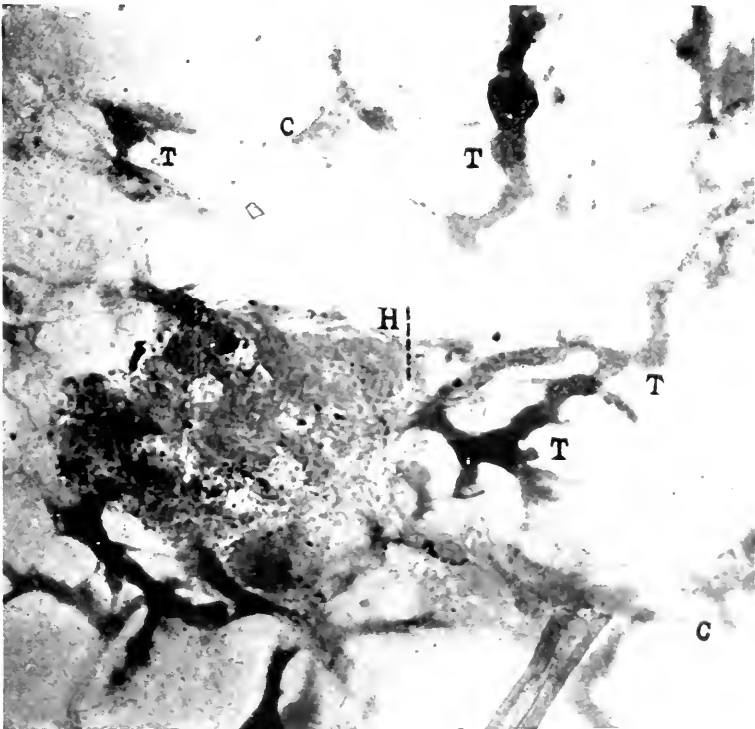


FIG. 2.

Smyth: Reactions between Bacteria and Animal Tissues.





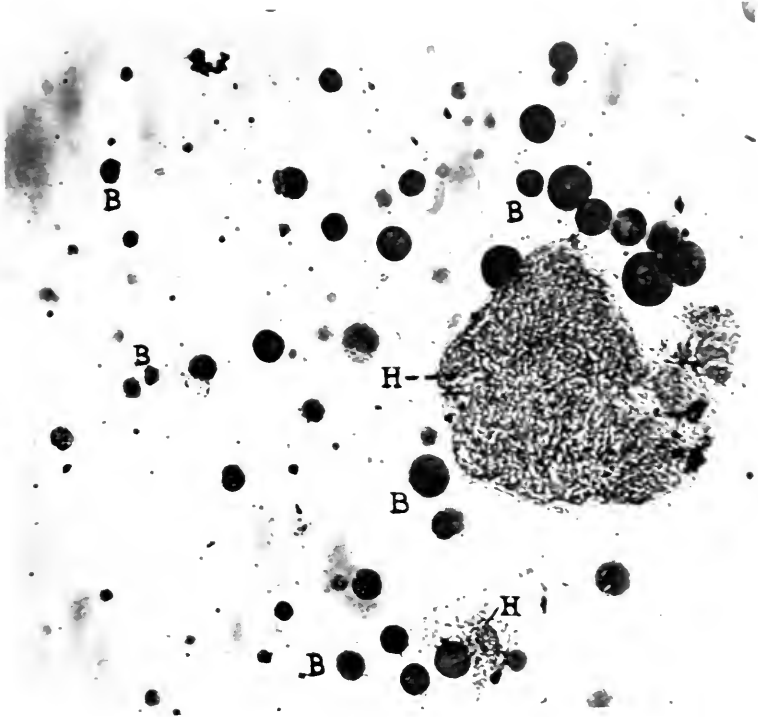


FIG.

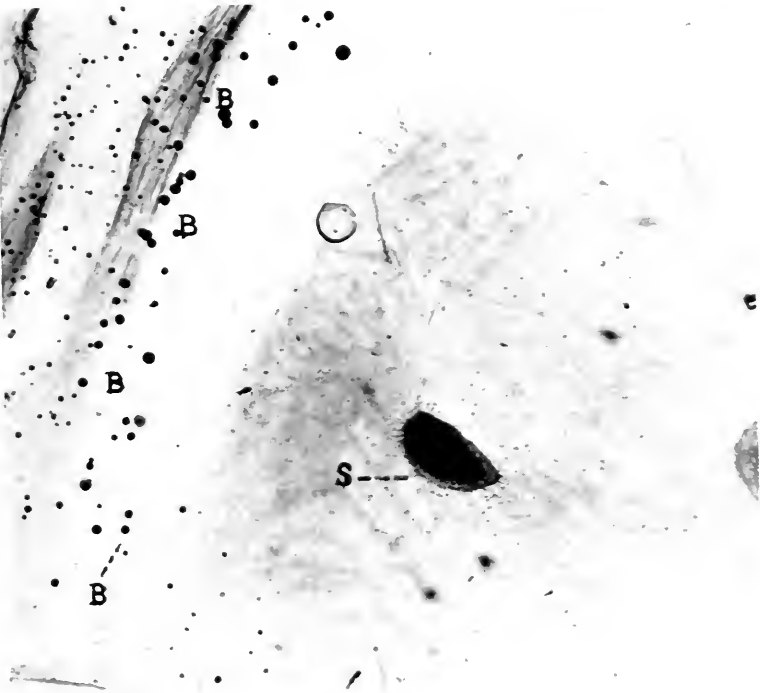


FIG. 2.  
Synthetic Reactions between Bacteria and Cells



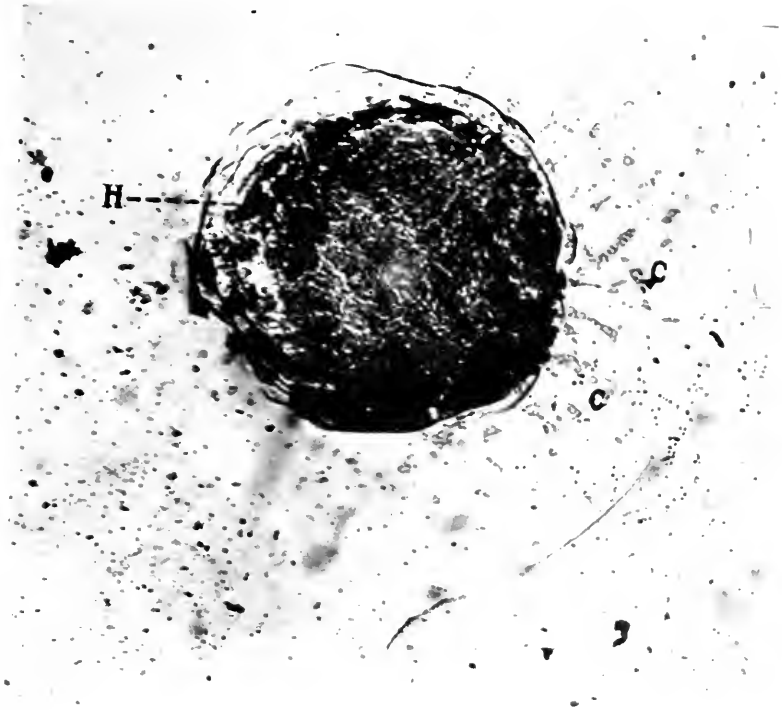


FIG. 5.



FIG. 6.

(Smyth: Reactions between Bacteria and Animal Tissues)



## PLATE 19.

FIG. 3. Twenty-four day culture from nine day chick embryo heart tissue in plasma inoculated with *Bacterium diphtheriticum* before planting. Notice the diphtheria colonies clustered around the tissue, with very little new tissue cell growth. Magnified 53 diameters. H = heart fragment. B = bacterial colonies.

FIG. 4. Two day culture from thirteen day chick embryo splenic tissue in plasma inoculated with *Bacterium diphtheriticum* before planting. Notice the large halo around the splenic tissue free from diphtheria colonies. The magnification is too small to show new tissue cells in this area. Magnified 12 diameters. S = splenic fragment. B = bacterial colonies.

## PLATE 20.

FIG. 5. Four day culture from seven day chick embryo heart tissue in plasma plus diphtheria toxin. Note the limited growth of highly inoculated cells. With higher magnification these cells show degenerated fragmenting nuclei. Magnified 53 diameters. H = heart fragment. C = new cell growth.

FIG. 6. Four day culture from seven day chick embryo heart tissue in plasma plus diphtheria toxin and antitoxin. (Same toxin dosage as in figure 5.) Note the nearly normal new growth of healthy cells. Magnified 53 diameters. H = heart fragment. C = new cell growth.

## VARIETIES OF PNEUMOCOCCUS AND THEIR RELATION TO LOBAR PNEUMONIA.\*

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In a previous paper Dochez and Gillespie<sup>1</sup> have shown that pneumococci derived from lobar pneumonia may be divided according to their immunological reactions into certain well defined groups. There developed from this study the fact that pneumococci fall into two general groups. The larger of these, consisting of about 80 per cent. of the strains encountered, can be further subdivided into smaller groups. The latter have been arbitrarily numbered groups I, II, and III, and it has been found that a single member of either group I or group II is characterized by the possession of immunity reactions identical with those of other strains of the homologous group. Group III consists of the type known as *Pneumococcus mucosus*, and the first classification of organisms into this group depended upon differences of morphology and cultural reaction. A subsequent study by Hanes<sup>2</sup> has shown that members of the *mucosus* group manifest cross agglutination, so that it has been possible to relate further the members of this group by means of at least one immunological test. The immunity reactions of these three groups apparently do not change on artificial cultivation or on animal passage.

The smaller of the two main groups, which has been named group IV, is peculiar in that it seems to consist of a series of independent varieties which do not cross in their immune reactions with members of groups I, II, or III, or with each other. This group represents about 20 per cent. of the strains obtained from cases of lobar pneumonia, and this frequency seems to be fairly constant from year to year.

In view of our ability to recognize by means of specific reactions

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<sup>1</sup> Dochez, A. R., and Gillespie, L. J., *Jour. Am. Med. Assn.*, 1913, lxi, 727.

<sup>2</sup> Hanes, F. M., *Jour. Exper. Med.*, 1914, xix, 38.

definite types of disease-producing pneumococci, we have deemed it important, in addition to confirming the constancy of these types, to study also the character of the pneumococci present in the normal human mouth, and on the mucous membranes of patients recovered from pneumonia, and to determine whether these strains differ in any way from those encountered during disease. If the type of organism that lives as a saprophyte on normal mucous membranes could be differentiated by certain fundamental and constant reactions from the ordinary disease-producing type, a reconstruction of our ideas concerning the epidemiology of lobar pneumonia would become necessary. Owing to the apparent lack of contact infection in pneumonia, investigators have been led to assume that the disease is due in most instances to infection with the pneumococcus that has been previously harbored in the mouth of the infected individual. If the strains present in normal mouths should show constant differences from the types found during actual disease, and if the latter types should be absent from the normal mouth flora, the likelihood would become great that infection in pneumonia takes place either by contact with infected individuals or apparently healthy carriers, however occult the steps which lead to infection may be.

During the past year we have been able to make a more extended study of the serological relationships of strains of pneumococci obtained from lobar pneumonia. The methods of classification have been the same as those previously employed; namely, agglutination and specific protection of animals against infection. Up to the present time no new group relationships have been discovered, and the recurrence of the types previously described has been constant.

In table I is shown the relative frequency of occurrence of organisms of the different types during the year 1912-13.

TABLE I.

	No.	Per cent.
Group I .....	35	47
Group II .....	13	18
Group III ( <i>mucosus</i> ) .....	10	13
Group IV (heterogeneous) .....	16	22
Total typical .....	58	78
Total heterogeneous .....	16	22
Total .....	74	

Table II shows a like classification of the strains obtained during 1913-14.

TABLE II.

	No.	Per cent.
Group I .....	21	30
Group II .....	28	39
Group III ( <i>mucosus</i> ) .....	6	8
Group IV (heterogeneous) .....	16	23
Total typical .....	55	77
Total heterogeneous .....	16	23
Total .....	71	

Tables I and II show the constancy with which the four groups described occur in two successive years. The percentage incidence of infection with members of the typical groups and with strains belonging to the heterogeneous group IV has been practically the same in both years. In 1912-13 the dominant type was group I. During 1913-14 the incidence of group I has diminished somewhat and there has been a corresponding rise in the number of infections with organisms belonging to group II.

The study of the various groups has brought out the interesting fact that the four types described differ in their degree of virulence for human beings. It has been impossible for us to obtain absolute figures of the mortality due to the different groups, because most of the individuals infected with groups I and II have been treated with specific antisera. In spite of the alteration in mortality brought about by this method of treatment, it has become clear that certain of the groups are more likely to cause a fatal infection than others. In general the severest forms of pneumonia result from infection with organisms belonging to groups II and III. The average virulence of group I seems to be somewhat lower, and, though infection with this type is usually severe in character, the mortality is definitely lower than in corresponding individuals infected with organisms belonging to groups II and III. The lowest grade of virulence is manifested by organisms of the heterogeneous group IV. Although infection with this group may run a severe course, it is unusual for it to terminate fatally.

These studies demonstrate the constancy with which the groups of pneumococcus described occur in New York City, and have now



been carried on for a sufficiently long period of time to render it unlikely that new groups closely related by immunity reactions will be encountered. In addition to these observations, investigations carried on by Dr. I. C. Walker,<sup>3</sup> in the Peter Bent Brigham Hospital, Boston, and by Dr. Paul Lewis,<sup>4</sup> in the Pennsylvania Hospital, Philadelphia, have shown that in these two cities the same groups of pneumococci are concerned in the causation of lobar pneumonia. Recently, by the use of immune sera prepared in this country, Professor Neufeld<sup>5</sup> has demonstrated the existence in Germany of groups of pneumococci having immune reactions identical with organisms belonging to our groups I and II. Previous study by Schottmüller<sup>6</sup> has already demonstrated the association of *Pneumococcus mucosus* with lobar pneumonia in Germany. Through the kindness of the South African Institute for Medical Research we have recently been able to test strains of pneumococcus isolated from cases of lobar pneumonia among natives in the Rand. The interesting observation has been made that even in this remote region of the world typical representatives of our groups I, II, and III are the causative agents in the production of lobar pneumonia. Lister<sup>7</sup> has described five groups of pneumococcus among the strains studied by him in South Africa. Three of these groups are identical with the groups met with in North America and in Germany. The other two groups, one of which appears to be dominant in South Africa, have not as yet been found in the cases of pneumonia studied by the writers. The possible significance of these two new races of pneumococcus will be discussed later in this paper.

STUDY OF STRAINS OF PNEUMOCOCCUS ISOLATED FROM THE MOUTHS  
OF NORMAL INDIVIDUALS.

Although there are in the literature instances of the apparent contagiousness of lobar pneumonia, it has been commonly assumed that most cases of the disease represent probable infection with a pneu-

<sup>3</sup> Personal communication.

<sup>4</sup> Personal communication.

<sup>5</sup> Personal communication.

<sup>6</sup> Schottmüller, H., *München. med. Wchnschr.*, 1903, 1, 909.

<sup>7</sup> Lister, F. S., *South African Institute for Medical Research [Publications]*, Dec. 22, 1913.

mococcus dwelling during health on the buccal mucous membrane of normal human beings. Owing to a sudden accession of virulence of the pneumococcus, or unusual depression of resistance of the individual, this organism is supposed to be able to penetrate the lungs and set up disease. In view of the constant relationship of certain definite groups of pneumococci to lobar pneumonia, an opportunity has been afforded to test the validity of this assumption.

Isolation of pneumococcus from the sputum of a large proportion of healthy individuals has been fairly easy. Care was taken to avoid persons in direct contact with cases of lobar pneumonia, and the organisms were obtained at a season when the incidence of the disease is at a low ebb. Organisms which did not fulfill the requirements necessary for identification of the pneumococcus were discarded unless retained for special reasons. Owing to the large number of tests necessary for sufficient comparison of the organisms, the study was limited to fifteen different strains obtained from separate sources. One of these belonged to the group of *Streptococcus viridans* and was carried along with the rest for purposes of comparison, and in order to see if any important change in character occurred in this organism during the period of manipulation. All organisms were first tested for agglutination and protection with sera developed from immunization of animals with standard members of pneumococcus groups I and II. Subsequently rabbits were immunized with the strains from normal sputum, and cross relationships between these strains were tested. The general result of the investigation has been to demonstrate that strains of pneumococcus isolated from normal sputum do not belong to any of the fixed groups of pneumococcus, *i. e.*, groups I, II, or III, but resemble, as far as we can determine, the heterogeneous group IV, which has been previously described. Of the fifteen strains examined, in thirteen the morphology was that of a typical lance-shaped diplococcus. Of the latter, eleven strains were typical pneumococci in every way, showing the characteristic encapsulation, bile solubility, cultural and fermentative reactions, and two showed certain variations such as the absence of capsules even after repeated observation under suitable conditions and a tendency to precipitate in broth media. Both of these strains were bile-soluble; one fermented, and the other failed

to ferment inulin. Of the two strains showing morphological variations one was *Streptococcus viridans*, and the other a bile-soluble, inulin-fermenting organism which morphologically and culturally resembled *Pneumococcus mucosus*. It produced a characteristic sticky exudate in the peritoneal cavity of infected animals, but repeated attempts failed to demonstrate clearly defined capsules. The virulence of the different strains varied from a relatively high degree to practical non-pathogenicity for white mice. On the whole, the virulence of the most typical organisms was greater than that of those presenting variations. Of the fifteen strains eleven were typical pneumococci, three were closely enough related to be included in the pneumococcus group, and one was *Streptococcus viridans*. The serum reactions of all the strains were tested in the manner described in a previous paper on the serological reactions of *Pneumococcus*. A study was first made of the power of immune sera derived from stock cultures of groups I and II to agglutinate and to protect against infection with the sputum strains. In preparing the agglutination reactions serum and twenty-four hour broth cultures were mixed in equal quantities in the tubes. Readings were made after two hours at 37° C. and twenty-four hours on ice. The results of the agglutination tests are shown in table III.

TABLE III.

*Agglutination of Sputum Strains with Stock Sera of Groups I and II.*

Culture of pneumococcus.	1		2		3		4		5		6		7		8		9		10		11		12		13		14		15	
	2	24	2	24	2	24	2	24	2	24	2	24	2	24	2	24	2	24	2	24	2	24	2	24	2	24	2	24	2	24
Group I.	0	0	+	++	0	0	0	0	0	+	0	0	0	+	0	+	0	+	0	0	0	0	0	0	0	0	0	+	0	±
Group II	0	0	0	0	0	0	0	0	0	+	0	0	0	0	0	0	0	0	0	0	0	0	0	0	+	+	+	0	+	0
Normal horse serum.	0	0	0	+	0	0	0	0	0	+	0	0	0	±	0	±	0	0	0	0	0	0	0	0	0	0	0	+	0	0

Of the fifteen strains presented in this table, only two showed agglutination with the type sera at the end of two hours. In all, eight strains showed variable degrees of agglutination at the end of twenty-four hours. Of these eight strains, five agglutinated in more than one of the sera employed in the tests, and in most instances the heterologous agglutination occurred in normal horse serum, thus showing a tendency of the sputum strains to undergo

spontaneous agglutination. Such a variation practically never occurs in strains of pneumococcus isolated from lobar pneumonia, all of which either show specific agglutination or, when this does not occur, remain uniformly suspended for twenty-four hours. Of the fifteen cultures tested, No. 13 was the only one that showed a characteristic specific agglutination in either of the type sera.

In the original study which led to a serological classification of the disease-producing pneumococci, protection of animals against infection was adopted as probably the most specific of the immunological tests which could be applied, and it was only later, when agglutination was found to correspond very closely in specificity with the protection tests, that it was also accepted as a satisfactory method of differentiation. In table IV are presented the results obtained by testing the protective power of type sera against sputum pneumococci. White mice were given intraperitoneally varying doses of pneumococci, and at the same time a fixed quantity of immune serum. Animals surviving for five days were considered effectively protected. All animals except the virulence controls received 0.2 of a cubic centimeter of immune serum intraperitoneally. Serum of type I, in quantities of 0.2 of a cubic centimeter, uniformly protects against 0.1 of a cubic centimeter of a broth culture of the homologous organism, which kills mice regularly in doses of 0.000001 of a cubic centimeter. The same amount of serum of type II protects against 0.01 of a cubic centimeter of the homologous organism of similar virulence. The specimen of type II serum used in the following tests had a somewhat lower protective value than usual, but was sufficiently high to show dependable differences.

The results of the protective tests with thirteen of the fifteen strains of cocci studied are presented in table IV. The two organisms not tested were of such low virulence that it was impossible to kill mice with the moderate doses necessary for the successful carrying out of the test. In no instance has the protective value of the type sera been sufficiently high to justify placing any of the organisms studied in either of the fixed groups I or II. The irregular survivals occurring with the smallest doses of culture are somewhat difficult to explain. As is observed, these survivals sometimes occur among the control animals, with one or both of the specific immune

TABLE IV.

*Protective Power of Type Sera for Pneumococci from Normal Sputum.*

Dose of culture.	Strains of pneumococcus.										
	1			2			3 ( <i>Pneumococcus mucosus</i> ).				
	Controls.	Serum I.	Serum II.	Controls.	Serum I.	Serum II.	Controls.	Serum I.	Serum II.	Normal horse serum.	
0.1 c.c.	.....	D. 14	D. 14	.....	D. 14	D. 14	.....	D. 20	D. 20	.....	
0.01 c.c.	D. 18	D. 18	D. 18	D. 14	D. 18	D. 18	D. 20	D. 20	D. 20	S.	
0.001 c.c.	.....	.....	.....	.....	.....	.....	D. 20	D. 20	D. 20(?)	S.	
0.0001 c.c.	D. 42	D. 18	D. 28	D. 28	S.	S.	S.	D. 48	D. 20	S.	
0.00001 c.c.	D. 96	D. 28	S.	D. 36	S.	S.	S.	S.	D. 84	S.	
	4			6			7				
	Controls.	Serum I.	Serum II.	Normal horse serum.	Controls.	Serum I.	Serum II.	Controls.	Serum I.	Serum II.	Normal horse serum.
0.1 c.c.	.....	D. 20	D. 20	.....	.....	D. 14	D. 18	.....	D. 20	D. 20	.....
0.01 c.c.	D. 20	D. 20	D. 20	D. 20	D. 18	D. 14	D. 18	D. 20	D. 20	D. 20	D. 20
0.001 c.c.	D. 20	D. 20	D. 20	D. 20	.....	.....	.....	D. 20	D. 20	D. 30	D. 20
0.0001 c.c.	D. 30	D. 20	D. 20	D. 20	D. 42	D. 24	D. 36	S.	D. 42	D. 20	D. 30
0.00001 c.c.	D. 42	D. 24	D. 30	D. 42	S.	D. 36	D. 42	S.	S.	D. 24	S.
	9				10			11			
	Controls.	Serum I.	Serum II.	Normal horse serum.	Controls.	Serum I.	Serum II.	Controls.	Serum I.	Serum II.	
0.1 c.c.	.....	D. 20	D. 20	.....	.....	D. 18	D. 18	.....	D. 18	D. 18	
0.01 c.c.	D. 20	D. 20	D. 30	D. 20	D. 18	D. 18	D. 18	D. 18	D. 18	D. 18	
0.001 c.c.	D. 20	D. 20	D. 30	D. 20	.....	.....	.....	.....	.....	.....	
0.0001 c.c.	S.	D. 36	D. 20	D. 30	D. 28	D. 18	D. 120	D. 72	D. 20	D. 20	
0.00001 c.c.	S.	D. 42	S.	D. 30	D. 36	D. 36	S.	D. 66	D. 28	D. 120	
	12				13						
	Controls.	Serum I.	Serum II.	Normal horse serum.	Controls.	Serum I.	Serum II.	Normal horse serum.			
0.1 c.c.	.....	D. 18	D. 18	.....	.....	D. 20	D. 20	.....			
0.01 c.c.	D. 18	D. 18	D. 18	.....	D. 20	D. 20	D. 20	D. 20			
0.001 c.c.	.....	.....	.....	.....	D. 20	D. 20	D. 20	D. 20			
0.0001 c.c.	D. 18	S.	D. 18	.....	D. 20	D. 3(?)	D. 24	D. 25			
0.00001 c.c.	D. 120	S.	S.	.....	D. 72	D. 34	S.	D. 20			
	14				15						
	Controls.	Serum I.	Serum II.	Normal horse serum.	Controls.	Serum I.	Serum II.	Normal horse serum.			
0.1 c.c.	.....	S.	D. 20	.....	.....	D. 20	D. 20	.....			
0.01 c.c.	S.	S.	S.	D. 20	D. 20	D. 20	D. 48	D. 20			
0.001 c.c.	S.	S.	S.	S.	D. 20	D. 20	D. 20	D. 20			
0.0001 c.c.	S.	S.	S.	S.	D. 30	D. 34	D. 20	D. 48			
0.00001 c.c.	S.	S.	D. 72	S.	D. 34	D. 34	D. 24	D. 132			

In the tables D.=died; S.=survived. The figures represent the number of hours before the death of the animal.

sera, and sometimes among the animals treated with normal horse serum. The average virulence of the sputum pneumococci for white mice is generally somewhat lower than that of pneumococci obtained from cases of lobar pneumonia. Our experience has been that among pneumococci of relatively low virulence variations in the effect of serum are often observed. In some instances the administration of serum seems to increase the infective power of the pneumococcus, and in other instances there may be a variable degree of protection that is apparently non-specific in character. Such effects are not manifest when highly virulent pneumococci from lobar pneumonia are used. For purposes of comparison a small number of such tests are shown in table V.

Table III shows that pneumococci 2, 9, 13, and 15 showed the nearest approach to specific agglutination, and we should expect some evidence of protective power with the corresponding sera, if the organisms in question belong definitely to the group in the sera of which agglutination occurred. Pneumococcus 2, which agglutinates slightly in serum I and strongly in serum II, shows a slight grade of protection with both sera; pneumococcus 9, which agglutinates slightly with serum I, shows no protection in either serum; pneumococcus 13, which develops strong agglutination only in serum II, shows a minimal degree of protection with the corresponding serum; pneumococcus 15 agglutinating slightly with serum I shows no protection with either of the type sera employed. These irregular crossings are not at present explainable, but they do not seem to indicate a close degree of specific relationship, such as is observed in the highly parasitic fixed types of pneumococcus. Raising the virulence when possible by animal passage does not change these organisms into typical representatives of any of the fixed types.

TABLE V.

*Agglutination of Fifteen Strains of Pneumococcus from Lobar Pneumonia with Stock Sera of Groups I and II.*

Culture of pneumococcus.	Group I.					Group II.					Group IV (heterogeneous).				
	A1	67	75	78	88	85	A3	A5	A10	A69	A38	A67	A71	A75	A78
Serum I. . . .	++	++	++	++	++	...	...	...	...	...	...	...	...	...	...
Serum II. . . .	...	...	...	...	...	++	++	++	+	++	...	...	...	...	...

Tables V and VI are presented to show how closely related are the strains of pneumococci obtained from lobar pneumonia to other members of the homologous group.

TABLE VI.

*Protective Power of Type Sera for Pneumococci from Lobar Pneumonia.*

Type of culture.	Strains of pneumococci of group I.														
	A1			67			75			78			88		
	Controls.	Serum I.	Serum II.	Controls.	Serum I.	Serum II.	Controls.	Serum I.	Serum II.	Controls.	Serum I.	Serum II.	Controls.	Serum I.	Serum II.
c.c.	D. 72	S.	D. 72	D. 17	S.	D. 41	.....	D. 120	.....	D. 18	D. 18	D. 18	.....	D. 18	.....
r c.c.	D. 144	S.	D. 96	D. 17	S.	D. 25	.....	S.	.....	D. 36	D. 20	D. 18	.....	S.	.....
or c.c.	S.	S.	S.	D. 41	S.	D. 41	.....	S.	.....	D. 23	S.	D. 18	.....	S.	.....
oor c.c.	D. 72	S.	D. 72	D. 41	S.	D. 41	D. 19	S.	D. 19	D. 19	S.	D. 20	D. 24	S.	D. 20
oooo c.c.	.....	.....	.....	D. 96	S.	D. 41	D. 30	S.	D. 24	D. 24	S.	D. 18	D. 20	S.	D. 22
ooooo c.c.	.....	.....	.....	D. 48	S.	D. 72	D. 30	S.	D. 40	D. 36	S.	D. 36	D. 40	S.	D. 40

	Strains of pneumococci of group II.														
	85			A3			A5			A10			A69		
	Controls.	Serum I.	Serum II.	Controls.	Serum I.	Serum II.	Controls.	Serum I.	Serum II.	Controls.	Serum I.	Serum II.	Controls.	Serum I.	Serum II.
c.c.	.....	.....	D. 18	.....	.....	.....	.....	.....	.....	.....	D. 18	D. 30	.....	D. 18	D. 18
r c.c.	.....	.....	S.	.....	.....	.....	.....	D. 18	S.	D. 19	D. 48	S.	.....	D. 18	S.
or c.c.	D. 18	D. 18	S.	D. 18	D. 18	D. 24	.....	D. 18	S.	D. 30	D. 36	S.	.....	D. 18	S.
oor c.c.	D. 18	D. 20	S.	D. 18	D. 18	S.	D. 30	D. 36	S.	D. 36	D. 48	S.	D. 18	.....	S.
oooo c.c.	D. 40	D. 22	S.	D. 18	D. 18	S.	D. 26	D. 18	S.	D. 36	D. 36	S.	D. 18	.....	S.
ooooo c.c.	D. 40	S.	S.	D. 18	D. 18	S.	D. 26	.....	S.	.....	.....	.....	D. 18	.....	S.

	Strains of pneumococci of group IV (heterogeneous).														
	A38			A67			A71			A75			A78		
	Controls.	Serum I.	Serum II.	Controls.	Serum I.	Serum II.	Controls.	Serum I.	Serum II.	Controls.	Serum I.	Serum II.	Controls.	Serum I.	Serum II.
c.c.	.....	D. 18	.....	.....	D. 18	.....	.....	D. 18	.....	.....	D. 18	.....	.....	D. 18	D. 18
r c.c.	.....	D. 24	.....	.....	D. 18	D. 24	.....	D. 18	D. 36	.....	D. 20	.....	.....	D. 20	D. 20
or c.c.	D. 20	D. 22	D. 20	D. 22	D. 30	D. 18	D. 20	D. 18	D. 18	D. 18	D. 20	D. 18	D. 18	D. 24	D. 24
oor c.c.	D. 48	D. 22	D. 48	D. 18	D. 36	D. 36	D. 25	D. 36	D. 25	D. 22	D. 21	D. 22	D. 22	D. 22	D. 22
oooo c.c.	D. 48	.....	D. 48	D. 36	.....	D. 36	D. 24	.....	D. 36	.....	.....	.....	.....	.....	.....
ooooo c.c.	D. 48	.....	D. 48	D. 36	.....	.....	D. 36	.....	.....	.....	.....	.....	.....	.....	.....

Strains of pneumococcus isolated from patients with lobar pneumonia, as has been previously demonstrated, can be classified into

definite groups by means of their serological reactions. In tables V and VI are presented the agglutination and protection reactions of a small number of these strains belonging to groups I, II, and IV. The reactions of group III, the *Pneumococcus mucosus* group, are not given, because they are developed with some difficulty and have been discussed elsewhere. It is seen that groups I and II correspond exactly in their agglutination and protection reactions, and show practically no crossing. In group IV have been placed those organisms which do not react with serum I and II, and which by their growth characters are differentiated from *Pneumococcus mucosus*. Previous study has shown that individual members of this group show very little crossing in their serological reactions, and in this they resemble closely the pneumococci isolated from normal sputum. In addition, they are, as a rule, less pathogenic than members of the fixed groups.

Comparison of immunological reactions of pneumococci from normal sputum and of those from lobar pneumonia reveals certain facts which may have an important bearing on the epidemiology of this disease. In about 80 per cent. of cases of pneumonia, pneumococci are found which fall into well defined serological groups, and these organisms do not occur in normal mouths except in exceptional cases, limited, as far as our experience goes, to intimate contacts. Some evidence is therefore offered in support of the assumption that infection with one of the fixed types of pneumococcus represents contact infection, either direct or indirect, from some previous case of pneumonia, and not infection with a pneumococcus which habitually dwells upon the mucous membranes. On the other hand, about 20 per cent. of cases of pneumonia are due to heterogeneous pneumococci which cannot be differentiated from the strains occurring in normal sputum. It is possible that these infections are autogenic in character.

In order to demonstrate still further the differences between pneumococci occurring in disease and in the normal mouth, we have studied the disappearance of the fixed types from the sputum during convalescence from pneumonia, and the character of the pneumococcus which then becomes manifest. This study is being extended at the present time, and the results of only a few observations are



here presented. The pneumococci studied during recovery were obtained from the sputum and differentiated by the tests previously described. Observations made at varying intervals after the onset of the disease are presented in table VII.

TABLE VII.

Case.	Type of pneumococcus during height of disease.	Type of pneumococcus after recovery.
W.R.	Type I	60 dys. Type I. 65 dys. Type IV.
A.W.	Type II	30 dys. Streptococcus. 48 dys. Type IV. 108 dys. Type IV.
A.	Type II	60 dys. Type IV.
S.H.	Type II	34 dys. Streptococcus. 40 dys. Type IV.
K.W.	Type III	13 dys. Type III. 73 dys. Type IV.
S.H.	Type II	47 dys. Streptococcus. 78 dys. Type IV.
S.	Type I	59 dys. Streptococcus. 73 dys. Streptococcus.
A.I.	Type II	53 dys. Type II.
McG.	Type I	30 dys. Type IV.
A.	Type I	90 dys. Type I.
U.	Type II	20 dys. Type IV. 25 dys. Type IV.
B.	Type I	33 dys. Type IV.
S.	Type I	30 dys. Type IV.
M.S.	Type II	30 dys. Type II.
C.	Type II	14 dys. Type IV.
B.	Type II	63 dys. Type II.
D.	Type II	21 dys. Type IV.
S.	Type II	24 dys. Type IV.
F.	Type I	12 dys. Type IV.
H.	Type II	15 dys. Streptococcus.

Study of the above table reveals the fact that only in exceptional instances does one find in the sputum, any considerable period of time after recovery, the type of pneumococcus with which the individual was infected during the disease. In four instances type organisms were still present at the time the patient was lost to observation, a period varying from thirty to ninety days after the onset of the disease. In two of these the signs of pneumonia persisted for a

long time and in one the type organism was obtained by lung puncture some three weeks after the onset of pneumonia. The pneumococcus can be isolated from the sputum of a large percentage of recovered cases, and this organism, except when the type strain has persisted, corresponds to the type of pneumococcus found in the mouths of normal individuals. The shortest period in which the type strain has been replaced by an organism of the sputum type has been twelve days after onset. Undoubtedly in a number of cases the first observations were made at too late a period to determine the exact time of disappearance of the type strain, and more carefully conducted studies are now showing that the type organisms disappear at an earlier period than table VII would lead one to suppose. The sputum type of pneumococcus obtained after recovery has been placed in what we term group IV, which consists, as has been stated previously, of a heterogeneous series of independent varieties according to our methods of classification. A systematic search for new fixed groups among these varieties is being carried on, but up to the present time no new group relationships have been discovered. From this study it is seen that the type organisms which are readily distinguishable during pneumonia are, in general, fairly rapidly supplanted by a pneumococcus which, as far as we can determine, corresponds to the type found in the sputum of normal individuals.

#### DISCUSSION.

As a result of the work described in this paper certain questions in regard to the etiology and epidemiology of lobar pneumonia present themselves for discussion. In order to produce disease, pathogenic microorganisms must gain entrance to the body through one of its exposed surfaces. Probably the commonest portal of entry is one of the mucous surfaces. It is well known that the mucous membranes of the body are constantly inhabited by a large variety of bacteria and that some of these microorganisms are closely related to certain strictly pathogenic types. Examples of such a condition are the Gram-negative, non-pathogenic diplococci, and the pathogenic and strictly parasitic gonococcus and meningococcus, also various bacilli of the gastro-intestinal group, and the typhoid bacillus. As a rule, the pathogenic members of these groups are readily dis-

tinguishable from their non-pathogenic relatives by a variety of simple reactions. Evidence is, however, gradually accumulating that the same condition of affairs exists among groups of organisms in which it is not so easy to differentiate the pathogenic from the non-pathogenic forms. It would seem probable that to this latter class belong the pneumococci, and that in this group we have strictly disease-producing types, and others which exist as more or less harmless saprophytes, which cannot be distinguished from the pathogenic members of the group, by the ordinary simple bacteriological methods. The studies reported in this paper show that about 80 per cent. of the pneumococci obtained from cases of lobar pneumonia belong to types which occur only in association with disease, and are not discoverable in the sputum of normal individuals. These seem to form the strictly parasitic types and are comparable to the true pathogens of certain other bacterial groups. In addition to these there are a smaller number of organisms associated with pneumonia which cannot be distinguished from the type of pneumococcus in normal sputum. Their lower virulence and general characters seem to indicate that they may be representatives of the type found in normal sputum. The more highly pathogenic forms are never found in normal sputum except in the case of intimate contacts. Whenever such contacts have been observed the type of pneumococcus in the contact has always corresponded exactly with that of the infected individual with whom he has been associated. These type organisms disappear from the mouths of contacts in the same manner as the same types disappear from the sputum of patients recovering from pneumonia.

It might be urged that the differences which we have demonstrated between various races of pneumococci are transient in character; that change from one type into another occurs with variation in environment. It is not possible at the present time to bring positive proof that such a phenomenon does not take place. Lyall<sup>8</sup> has recently isolated the pneumococci present in the deep sputum of a series of cases of pulmonary tuberculosis. In most instances these resembled the pneumococcus found in normal sputum. If change from the sputum type into the pathogenic types occurs with any

<sup>8</sup> Lyall, H. W., *Jour. Exper. Med.*, 1915, xxi, 146.

degree of readiness, one would expect to observe such changes under the conditions of this study, since in pulmonary tuberculosis the pneumococcus lives in the lung under conditions closely resembling those of lobar pneumonia. As our experience increases, we are more and more impressed with the constancy with which the varieties of pneumococcus retain their differential characters. No amount of animal passage, growth under a great variety of artificial conditions, or storage in a dried state has as yet caused the change of one type of pneumococcus into that of another, or the loss of the special characters by means of which it was originally classified. That the pneumococcus in its natural environment has reached a certain stage of equilibrium is fairly certain. In our experience we always obtain the same type of pneumococcus from normal sputum, and the same variety of groups from cases of lobar pneumonia. The pathogenic groups are now known to occur over widely separated areas of the globe. The status of bacterial mutation is so unsettled that in the absence of positive examples of permanent variations it has not seemed useful to us at the present time to make a study of the possibility of such changes among the pneumococci.

In view of the differences which seem to exist between pneumococci found in normal mouths and the dominant types occurring in lobar pneumonia, there is some interest in seeking an explanation of how they may be related to each other, and how the parasitic type has arisen from the more commonly saprophytic varieties. One might assume that today certain strains of pneumococcus are changing from more or less harmless organisms of the mouth to the disease-producing type found in pneumonia. The fact that a certain percentage of the organisms found in disease correspond more or less closely to those living in the normal mouth is some evidence in favor of this assumption. However, the majority of pneumococci which produce disease differ sharply from the type of organism found in normal sputum, and no new groups consisting of a large number of closely related strains have been found. If, then, the sputum type of pneumococcus rises to a high plane of parasitism, which enables it to produce a typical lobar pneumonia, it would seem to lose this quality after a single instance of infection, and not be able to perpetuate itself as a permanently pathogenic form and thus give rise to a number of instances of the disease.

We have recently received some strains of pneumococcus from South Africa, which seem to throw light on the manner in which new typical groups of pathogenic pneumococci arise, and the conditions that are necessary for these organisms to maintain their newly acquired disease-producing qualities. For a number of years pneumonia has been epidemic among the native mine laborers on the Rand. These men come for the most part from the more tropical regions of Africa where pneumonia is practically unknown, and they may be assumed to have a high degree of susceptibility to pneumococcus infection. The studies of Sir Almroth Wright<sup>9</sup> lend support to this assumption. As soon as the natives are brought to the Rand and come in contact with whites among whom pneumonia is fairly common, a high rate of incidence of pneumonia immediately develops among the blacks. Lister<sup>10</sup> has studied the strains of pneumococci here concerned and finds that they fall into five different groups showing specific immunological reactions. He has sent us five typical representations of these groups for comparison with the groups of pneumococci occurring in this country. Three of the strains give the characteristic reactions of members of the three typical groups found in New York. Two of the strains which belong to the dominant groups in South Africa have not as yet been met with here, and in view of the large number of organisms that have been examined by us, and from Neufeld's experience in Germany, it seems that they must be peculiar to South Africa. In other words, the condition in the Rand has been favorable to the origin of new fixed groups of pneumococcus. The most likely explanation of this phenomenon seems to be that in South Africa, among the whites, as in this country, there are a number of instances of pneumonia due to organisms resembling the sputum pneumococcus, the slightly virulent group IV of our classification. When such a pneumococcus is communicated to the susceptible black its pathogenic history, in spite of its relatively low virulence, does not stop with the production of a single instance of pneumonia, as it seems to among individuals whose racial immunity is relatively high, but the organism is readily passed on to other susceptible blacks, and

<sup>9</sup> Wright, A., *Lancet*, 1914, ii, 1, 87.

<sup>10</sup> Lister, F. S., *loc. cit.*

thus establishes itself in the less immune race as a permanently pathogenic type. If such is in reality the succession of events, then it is probable that permanent parasites arise only under conditions of high racial susceptibility, and that as the immunity of the race develops the ground becomes unfavorable for the occurrence of such a phenomenon, and no more new fixed races appear. Under the latter conditions the organisms of low pathogenicity may produce isolated instances of disease among especially susceptible individuals, but because of their low virulence and the increased racial immunity they can no longer establish themselves as permanently parasitic types. Where relative immunes are brought into contact with non-immunes, the condition, as far as the origin of new disease-producing races is concerned, is much the same as when the whole race is highly susceptible. It is possible that in South Africa the new races arising among the natives may after a number of generations gain sufficient virulence to become highly infectious for the relatively immune white.

#### SUMMARY.

A study of pneumococci isolated from individuals suffering from lobar pneumonia has shown that the majority of these organisms fall into definite biological groups. These groups have been arbitrarily numbered from I to IV. The first three groups consist of organisms which within the group are closely related to each other by certain immunological reactions; *i. e.*, protection and agglutination. Extensive study has failed to reveal crossing in either of these reactions between members of separate groups. The fourth group is formed of a series of independent varieties which cannot be definitely related to one another by the immune reactions employed. Up to the present time we have observed no tendency of these organisms to lose their specific characters, nor have we observed a change of one type into another. These groups vary in their pathogenicity for human beings, and in the order of their virulence are as follows: group III, group II, group I, group IV. The degree of protective power developed in the sera of animals immunized against members of the different groups varies inversely with the virulence and with the amount of capsular development. This, however, applies only

to tests of passive immunity. The highly virulent groups give as good active immunity as those of lower virulence, if not better.

In view of these constant differential characters of the pneumococcus, it was deemed advisable to study the pneumococci occurring in normal sputum. It has been commonly assumed that infection in pneumonia is autogenic, and occurs from the invasion of the lungs by a pneumococcus habitually carried in the mouth. If this is so, we should find the same types in the normal mouth as occur during the disease. Examination of a series of normal individuals showed this not to be the case. In no instance was an organism found which could be grouped with any of the fixed types of pneumococcus. All exhibited the same characters as those organisms obtained from lobar pneumonia which belong to group IV. Inasmuch as organisms belonging to this group are of low virulence, and are responsible in our experience for only 20 per cent. of the cases of pneumonia, it is at once manifest that the majority and more virulent cases of pneumonia are due to organisms which are not found in normal mouths. To gain further evidence of this difference, a study has been made of convalescents from pneumonia who had been infected by typical organisms. During the period of recovery these typical organisms are supplanted by the type which occurs in normal mouths. The period of disappearance of the typical varieties has varied. The shortest time in which disappearance has occurred has been twelve days, and the longest period in which typical organisms have been carried has been ninety days. In the latter instance the patient was lost sight of, so that he may well have carried the virulent form for a longer period of time. In general, when typical organisms persist for a long time, there is delay in the healing of the lung lesion. If recovery is prompt, as a rule the virulent types disappear rapidly.

We have said that the virulent types do not occur in normal mouths. There are exceptions to this observation. In a number of instances organisms belonging to the typical groups have been isolated from the mouth sputum of healthy individuals. So far this has occurred only in individuals intimately in contact with cases of lobar pneumonia. Wherever typical organisms have been obtained under such circumstances, the type has always corresponded to that with which the case of pneumonia was infected. Such individuals,

therefore, become infected with virulent types of pneumococcus by contact, and may be regarded as healthy carriers of disease-producing types.

This study makes it probable that the majority of cases of pneumonia are dependent upon either direct or indirect contact with a previous case. Mere infection of the mouth by virulent types is by no means sufficient to cause the disease. In order to invade the lungs, these virulent types must find the circumstances favorable, or a suitable condition must arise during the period when they are harbored in the mouth. Comparative study of certain strains of pneumococci received from South Africa suggests that new groups of parasitic organisms develop only during the period of high racial susceptibility. A like condition of affairs is brought about when a group of hitherto unexposed individuals is brought into contact with an infectious microörganism. The development of racial immunity soon limits the number of new types which may arise.

The suggestion is made that strictly parasitic races of microorganisms are pure lines and have established themselves as parasites during a period of high racial susceptibility.



## THE DISTRIBUTION OF THE IMMUNE BODIES OCCURRING IN ANTIPNEUMOCOCCUS SERUM.\*

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By a systematic study of the specific antigenic properties of the pneumococcus, Dochez and Gillespie<sup>1</sup> have demonstrated four distinct types of this organism occurring in disease. Such a biologic classification of pneumococci affords the only rational basis for the clinical application of serum therapy in lobar pneumonia. The immunologic and clinical studies of Cole<sup>2</sup> have shown that the protective and curative value of antipneumococcus serum is dependent upon this group specificity, that a serum to be efficacious must be one produced in response to an organism of the same type as that causing the infection. The treatment by specific antisera of lobar pneumonia of pneumococcus origin is at present possible only in infections due to organisms of types I and II. Immune serum produced by the third group, *Pneumococcus mucosus*, fails to confer passive immunity, and the heterogeneous nature of organisms of the fourth variety demands a specific serum for each individual strain, making serum therapy in infections of this type impracticable. However, since approximately 70 per cent. of the cases of this disease is due to organisms of the first two groups, the serum treatment of pneumonia is applicable in the majority of instances. Experimental evidence and clinical experience have demonstrated the necessity of administering relatively large doses of the appropriate serum in combating these infections. The present study was undertaken, therefore, with the hope of determining a method of concentrating and purifying antipneumococcus serum, by which its antibacterial potency might be conserved with a minimum of foreign protein. It seems reasonable to assume that such a process, by concentration of antibody content, might enhance the efficacy of

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<sup>1</sup> Dochez, A. R., and Gillespie, L. J., *Jour. Am. Med. Assn.*, 1913, lxi, 727.

<sup>2</sup> Cole, R., *Arch. Int. Med.*, 1914, xiv, 56.

serum treatment, and, by the elimination of certain irrelevant protein constituents, lessen the incidence, or at least ameliorate the symptoms of serum disease.

Although much is known concerning the immunological reactions of various immune bodies, comparatively little is understood of their true chemical nature. Whether they are themselves protein in character, or merely associated in some obscure chemical combination with the globulin or albumin of immune sera is not definitely known. The fact that they may be thrown out of solution by certain protein precipitants has been utilized in the concentration of antitoxic sera. Most investigators now agree that antibody precipitation by chemical agents is not merely a mechanical or adsorption phenomenon, but that these immune substances partake of the nature of the protein with which they are precipitated.

In the present study it was first necessary, therefore, to determine with which fraction of the serum protein the pneumococcus immune bodies are associated. The serum used in these experiments was obtained by the intensive immunization of horses to virulent cultures of pneumococcus of types I and II. The various protein fractions isolated were tested for antibacterial action against virulent cultures of pneumococcus by protection experiments on white mice, and the results compared with the similar power of the whole serum. This method is less accurate than the more exact titration of antitoxic sera. The potency of antipneumococcus serum can be measured only by its ability to protect highly susceptible animals against actual infection, and the comparative antibody content of any given serum fraction can be interpreted only in terms of the death or survival of such animals. This reaction between antibody and bacterium is biological and not chemical, as in the neutral balance of antitoxin and toxin mixtures. Under these experimental conditions, however, some information has been gained concerning the distribution of the immune bodies occurring in antipneumococcus serum.

*Experiment 1.*—To determine the protective value of the total globulins precipitated by half saturation with ammonium sulphate.

300 c.c. of antipneumococcus serum I were diluted with an equal volume of water, and 600 c.c. of a saturated solution of ammonium sulphate were added.

The mixture was allowed to stand at room temperature over night, filtered, the precipitate collected, pressed, and dialyzed for eight days against running tap water. To the filtrate crystals of ammonium sulphate were added to full saturation. The precipitate consisting of albumin was filtered off, pressed, and dialyzed as above.

The volume of globulin solution was 100 c.c., so that the globulins were three times as concentrated as in the whole serum. The volume of albumin solution was 58 c.c., being 0.2 per cent. that of the original serum.

*Protective Power of Globulins, Albumin, and Serum I.  
Pneumococcus I.*

Amount of culture I.	Amount of serum fractions.	Serum I.	Globulins.	Albumin.	Controls, Culture alone.
0.05 c.c.	0.1 c.c.	S.	S.	D. 18	.....
0.05 c.c.	0.08 c.c.	S.	S.	D. 18	.....
0.05 c.c.	0.05 c.c.	S.	S.	D. 18	.....
0.05 c.c.	0.03 c.c.	D. 72	S.	D. 18	.....
0.05 c.c.	0.01 c.c.	D. 48	D. 72	D. 18	.....
0.0001 c.c.	.....	.....	.....	.....	D. 36
0.00001 c.c.	.....	.....	.....	.....	D. 36
0.000001 c.c.	.....	.....	.....	.....	D. 36

In the tables D.=died; S.=survived. The figures represent the number of hours before the death of the animal.

*Experiment 2.*—Antipneumococcus serum II was used, diluted with an equal volume of water, and the globulins were precipitated by half saturation with ammonium sulphate. The albumin was precipitated from the filtrate by complete saturation with crystals of ammonium sulphate. Both fractions were dialyzed for four days against running tap water, and made up to the original volume of serum with 0.85 per cent. salt solution.

*Comparative Protective Value of Globulins, Albumin, and Serum II.  
Pneumococcus II.*

Amount of culture II.	Amount of serum fractions.	Serum II.	Globulins.	Albumin.	Controls, Culture alone.
0.1 c.c.	0.2 c.c.	D. 66	D. 66	D. 18	.....
0.01 c.c.	0.2 c.c.	S.	S.	D. 19	.....
0.001 c.c.	0.2 c.c.	S.	S.	D. 19	.....
0.0001 c.c.	0.2 c.c.	S.	S.	D. 24	D. 19
0.00001 c.c.	0.2 c.c.	S.	S.	D. 24	D. 24
0.000001 c.c.	0.2 c.c.	S.	S.	D. 36	D. 24

*Agglutination.*

Dilution.	Serum II.	Globulins.	Albumin.	Controls.
I : I	++	++	—	—
I : 10	++	++	—	—
I : 20	+	±	—	—
I : 40	—	—	—	—

*Experiment 3.*—The agglutination test was made by mixing equal volumes of an eighteen hour broth culture of pneumococcus I and varying dilutions of serum fractions.

The antigen used in the precipitin reaction was prepared from the washed bacterial residue of liter cultures of pneumococcus I frozen and desiccated in vacuum over sulphuric acid. The dry powder was dissolved in salt solution, with 1 mg. per c.c., and a 1:10 dilution of this was used in the test. Equal parts of antigen and serum dilutions were mixed and incubated in the water bath at 37° C. for two hours.

*Agglutination and Precipitation.*

Agglutination.				Precipitation.		
Serum dilution.	Serum I.	Globulins.	Albumin.	Serum dilution.	Serum I.	Globulins.
I : I	++	++	—	I : 10	++	++
I : 10	++	++	—	I : 15	++	++
I : 100	—	—	—	I : 20	++	+
				I : 25	+	±
				I : 30	±	—

From experiments I, II, and III it may be concluded that the immune bodies of antipneumococcus serum are not dialyzable, that they are precipitated with the globulins by half saturation with ammonium sulphate, and that the demonstrable antibodies, such as agglutinins, precipitins, and protective substances, are combined or associated with the globulins and not with the albumin of the immune serum.

*Experiment 4.*—To determine the protective value of the euglobulin and pseudoglobulin.

300 c.c. of antipneumococcus serum I were diluted with an equal volume of water and 600 c.c. of a saturated solution of ammonium sulphate added, allowed to stand over night, and filtered, precipitate (a), filtrate (a). The precipitate (a) containing both globulins was taken up in 600 c.c. of water and saturated with crystals of sodium chloride (210 gm.) and filtered, precipitate (b), filtrate (b). The precipitate (b) was again taken up in water and resaturated with salt, the final precipitate pressed and dialyzed against running tap water until salt-free. The dialysate was made slightly alkaline to effect solution, used undiluted, and called "euglobulin." To the filtrate (b) about 0.2 per cent. of acetic acid was added to precipitate the globulin remaining in solution. This precipitate was pressed and after dialyzing for twenty-four hours was neutralized to litmus by sodium carbonate, and dialysis continued for four days. The dialysate was used undiluted and called "pseudoglobulin." The original filtrate (a) was saturated with crystals of ammonium sulphate; the precipitate containing the albumin was collected by filtration, pressed, and dialyzed.

*Comparative Protective Value.*

Amount of culture I.	Amount of serum fractions.	Serum I.	Euglobulin.	Pseudoglobulin.	Albumin.	Controls.
0.1 c.c.	0.2 c.c.	D. 72	D. 17	D. 20	D. 17	.....
0.01 c.c.	0.2 c.c.	S.	S.	D. 84	D. 17	.....
0.001 c.c.	0.2 c.c.	S.	S.	S.	D. 20	.....
0.0001 c.c.	0.2 c.c.	S.	S.	S.	D. 24	D. 22
0.00001 c.c.	.....	.....	.....	.....	.....	D. 24
0.000001 c.c.	.....	.....	.....	.....	.....	D. 36

Amount of culture I.	Amount of serum fractions.	Serum I.	Euglobulin.	Pseudoglobulin.	Albumin.	Controls.
0.01 c.c.	0.4 c.c.	S.	S.	S.	D. 48	.....
0.01 c.c.	0.3 c.c.	S.	S.	S.	D. 36	.....
0.01 c.c.	0.2 c.c.	S.	S.	S.	D. 18	.....
0.01 c.c.	0.1 c.c.	S.	S.	S.	D. 28	.....
0.01 c.c.	0.01 c.c.	D. 40	D. 40	D. 40	D. 18	.....
0.01 c.c.	0.005 c.c.	D.	D. 18	D. 48	D. 18	.....
0.0001 c.c.	.....	.....	.....	.....	.....	D. 18
0.00001 c.c.	.....	.....	.....	.....	.....	D. 28
0.000001 c.c.	.....	.....	.....	.....	.....	D. 36

Experiment 4 shows that by the technique employed the immune bodies of antipneumococcus serum are not confined to the so called euglobulin or pseudoglobulin, but occur in both these fractions. Their absence in the albumin of the serum confirms the previous experiment.

*Experiment 5.*—Fractional precipitation of heated serum by ammonium sulphate.

50 c.c. of antipneumococcus serum I were diluted with a half volume of distilled water and 1 per cent. sodium chloride, and 38.6 c.c. of a saturated solution of ammonium sulphate were added, making the total 34 per cent. saturation. The serum-sulphate mixture was heated in a water bath at 56° C. for four hours, 58° C. for one hour, and 60° C. for five minutes. Filtered hot, the precipitate was pressed and dialyzed for four days against running water. To the filtrate, a saturated solution of ammonium sulphate was added up to 54 per cent. saturation. After standing at room temperature over night the precipitate was filtered off, pressed, and dialyzed for four days. To the final filtrate ammonium sulphate crystals were added to complete saturation, and the filtrate was pressed and dialyzed. The various fractions isolated were designated 34 per cent. globulin, 54 per cent. globulin, and albumin, respectively. After dialysis these fractions were each made up with 0.85 per cent. salt solution to the volume of the original serum, and in the case of the 34 per cent. globulin sufficient sodium carbonate was added to effect solution.

*Comparative Protective Value of Globulin Fractions Precipitated by Ammonium Sulphate from Heated Serum.*

Amount of culture I.	Amount of serum fractions.	0-34% globulin.	34-54% globulin.	Albumin.	Whole globulins, experiment 1.	Serum I.	Controls, Culture alone.
0.1 c.c.	0.2 c.c.	D. 20	D. 20	D. 20	S.	S.	.....
0.01 c.c.	0.2 c.c.	S.	S.	D. 20	S.	S.	.....
0.001 c.c.	0.2 c.c.	S.	S.	D. 20	S.	S.	.....
0.0001 c.c.	0.2 c.c.	S.	S.	D. 20	S.	S.	D. 18
0.00001 c.c.	0.2 c.c.	S.	S.	D. 24	S.	S.	D. 18
0.000001 c.c.	0.2 c.c.	S.	S.	D. 24	S.	S.	D. 18

Agglutination tests showed the presence of these antibodies in both fractions, and their absence in the albumin.

The method followed in this experiment is essentially that devised by Banzhaf<sup>3</sup> for the concentration of diphtheria antitoxin. In diphtheria immune serum the antitoxin is precipitated with the pseudoglobulin. The addition of salt and the heating of serum-sulphate mixture converts some of the pseudoglobulin into the inactive euglobulin without material loss of its antitoxic potency. By the application of this method to antipneumococcus serum it was hoped that the residue of protective antibodies occurring in the pseudoglobulin might be changed over with the converted euglobulin and the total potency of the serum be thus concentrated in the latter fraction. This, however, did not occur; both fractions still showed protection. Heating antipneumococcus serum to the temperature used in this experiment does not seem materially to affect the activity of the agglutinins and protective antibodies, although some loss of potency is suffered by the latter.

*Experiment 6.*—To determine the protective value of the globulin fraction precipitated by passage of carbon dioxide through diluted serum.

50 c.c. of antipneumococcus serum II were diluted with ten volumes of distilled water. Carbon dioxide was allowed to bubble through slowly for two hours, the diluted serum being kept at a temperature of 2° C. during the process; it was then placed in the ice box over night, and a sharp separation of precipitate and supernatant fluid was effected by centrifugalization. The carbon dioxide precipitate was dissolved in 50 c.c. of 0.85 per cent. salt solution. The supernatant fluid was half saturated with ammonium sulphate by the addition of an equal volume of a saturated solution of this salt. This carried down the residual globulin remaining after removal of the carbon dioxide-insoluble

<sup>3</sup> Banzhaf, E. J., *Studies from the Research Laboratory, Department of Health*, New York, 1912-13, vii, 114.

fraction. After standing at room temperature over night the precipitate was filtered, pressed, and dialyzed for four days and made up to a volume of 50 c.c. with 0.85 per cent. salt solution.

*Protective Value of Carbon Dioxide Globulins.*

Amount of culture II.	Amount of serum fractions.	Original serum II.	Carbon dioxide-insoluble globulin.	Carbon dioxide-soluble globulin.	Controls, Culture alone.
0.1 c.c.	0.2 c.c.	D. 36	D. 36	D. 18	.....
0.01 c.c.	0.2 c.c.	S.	S.	S.	.....
0.001 c.c.	0.2 c.c.	S.	S.	S.	.....
0.0001 c.c.	0.2 c.c.	S.	S.	S.	D. 20
0.00001 c.c.	0.2 c.c.	S.	S.	S.	D. 18
0.000001 c.c.	0.2 c.c.	S.	S.	S.	D. 18

*Agglutination.*

Dilution of serum fractions.	Original serum II.	Carbon dioxide-insoluble globulin.	Carbon dioxide-soluble globulin.
1 : 1	++	++	++
1 : 5	++	++	+
1 : 10	+	+	—
1 : 15	±	—	—
1 : 25	—	—	—

*Precipitation.*

Antigen 1:1,000.

Dilution of serum fractions.	Original serum II.	Carbon dioxide-insoluble globulin.	Carbon dioxide-soluble globulin.
1 : 1	++	++	+±
1 : 5	+	+	—
1 : 10	±	± (?)	—

From experiment 6 it appears that from diluted antipneumococcus serum there is precipitated by saturation with carbon dioxide a globulin possessing high protective value, and that by the method of titration employed a slightly less potent globulin fraction remains in solution, and can be separated from the albumin by ammonium sulphate. The somewhat anomalous fact, that each fraction of the carbon dioxide globulin apparently possesses protective value equal to the whole serum, may be explained in part by the method of titration. The immunity unit, 0.2 of a cubic centimeter of serum, which has been found most suitable for protection tests, apparently contains an excess of antibody and the zone of carbon

dioxide precipitation may so lie within the limits of this excess that, in the cleavage effected by acidification with carbon dioxide, sufficient antibody is carried over with each fraction of the globulin to afford an equivalent protection. The carbon dioxide-insoluble globulin, although representing approximately only one-fifteenth of the total protein, apparently contains more than half the antibodies.

*Experiment 7.*—To determine the potency of the serum globulins soluble and insoluble in saturated sodium chloride.

50 c.c. of antipneumococcus serum II diluted with an equal volume of water were saturated with crystals of sodium chloride, and the salt-serum mixture was allowed to extract over night at room temperature. The portion of the globulin insoluble in saturated sodium chloride was filtered off, pressed, and dialyzed for four days against running water. To the filtrate containing the salt-soluble fraction was added an equal volume of a saturated solution of ammonium sulphate. The precipitate containing the salt-soluble globulin was collected, pressed, and dialyzed for four days. The fractions were all diluted with 0.85 per cent. salt solution to the volume of the original serum.

*Protective Value of Sodium Chloride Globulins.*

Amount of culture II.	Amount of serum fractions.	Original serum II.	Sodium chloride-insoluble globulin.	Sodium chloride-soluble globulin.	Controls. Culture alone.
0.01 c.c.	0.2 c.c.	S.	D. 20	S.	.....
0.001 c.c.	0.2 c.c.	S.	S.	S.	.....
0.0001 c.c.	0.2 c.c.	S.	S.	S.	D. 20
0.00001 c.c.	0.2 c.c.	S.	S.	S.	D. 24
0.000001 c.c.	0.2 c.c.	S.	S.	S.	D. 36

*Agglutination.*

Serum II .....	++
Sodium chloride-insoluble .....	++
Sodium chloride-soluble .....	++

Experiment 7 shows that the protective substances and the agglutinins of antipneumococcus serum are not confined to either of the globulin fractions separated by saturation with sodium chloride. The salt-insoluble globulin, according to Freund and Joachim,<sup>4</sup> represents the euglobulin, but includes only a part of the similar fraction precipitated by one-third saturation with ammonium sulphate. Banzhaf and Gibson<sup>5</sup> consider that this method yields as

<sup>4</sup> Freund, E., and Joachim, J., *Ztschr. f. physiol. Chem.*, 1902, xxxvi, 407.

<sup>5</sup> Banzhaf, E. J., and Gibson, R. B., *Studies from the Research Laboratory, Department of Health, New York*, 1908-10, iv-v, 202.



sharp a differentiation as possible between the two globulin fractions, and that the sodium chloride separation probably represents more truly the common conception of euglobulin and pseudoglobulin than the more usual fractioning by ammonium sulphate. The results obtained in this experiment are similar to and comparable with those of experiment 4, in which the globulins were first precipitated with ammonium sulphate, and then separated by precipitation first with sodium chloride and then with acetic acid.

*Experiment 8.*—To determine the potency of the water-soluble and -insoluble serum globulin obtained by dialysis.

100 c.c. of antipneumococcus serum II, undiluted, were placed in a parchment bag and dialyzed for six days against running tap water. The dialyzing bag was then washed out with distilled water, the precipitate collected by filtration, washed with water, taken up in 100 c.c. of physiological salt solution, and made slightly alkaline with sodium carbonate to effect complete solution. To the filtrate containing the water-soluble globulin, an equal volume of a saturated solution of ammonium sulphate was added, the filtrate collected, pressed, and dialyzed for four days, and then made up to volume (100 c.c.) with 0.85 per cent. salt solution.

*Protective Value of Globulin Fractions Separated by Dialysis.*

Amount of culture II.	Amount of serum fractions.	Water-soluble globulin.	Water-insoluble globulin.	Original serum II.	Controls.
0.01 c.c.	0.2 c.c.	D. 24	S.	S.	.....
0.001 c.c.	0.2 c.c.	S.	S.	S.	.....
0.0001 c.c.	0.2 c.c.	S.	S.	S.	D. 20
0.00001 c.c.	0.2 c.c.	S.	S.	S.	D. 24
0.000001 c.c.	0.2 c.c.	S.	S.	S.	D. 40

*Agglutination.*

Pneumococcus II.	Serum II.	Water-insoluble globulin.	Water-soluble globulin.
	++	++	++

Seng<sup>6</sup> showed that by dialysis of diphtheria antitoxin only a small part (1/23 to 1/11) of the total globulin was insoluble and that this fraction possessed no antitoxic value. These results were later confirmed by Brieger and Krause.<sup>7</sup> Freund and Joachim do not consider the globulins precipitated by fractionation with ammonium sulphate identical with the two groups obtained by dialysis.

<sup>6</sup> Seng, W., *Ztschr. f. Hyg. u. Infektionskrankh.*, 1899, xxxi, 513.

<sup>7</sup> Brieger, L., and Krause, M., *Berl. klin. Wchnschr.*, 1907, xlv, 946.

Experiment 8 demonstrates the distribution of the antibacterial substances of antipneumococcus serum in both fractions of the globulins separated by dialysis. These results indicate that the globulin precipitated by dialysis (water-insoluble), like that precipitated by carbon dioxide, contains a part, but not all of the immune bodies.

*Experiment 9.*—To determine the relative potency of the globulin fractions obtained by the addition of progressive amounts of ammonium sulphate.

50 c.c. of antipneumococcus serum II were diluted with a half volume of water and the whole was precipitated by 34 per cent. saturation with ammonium sulphate, 38.6 c.c. of saturated solution. From the filtrate the residual globulin was precipitated by adding sufficient saturated solution of ammonium sulphate to cause 54 per cent. saturation, allowing for the amount of this salt already present in the filtrate. The precipitates of both fractions were collected, pressed, and dialyzed for four days against running tap water. The dialysates were made up to the original volume of the serum with 0.85 per cent. salt solution, and were designated 0 to 34 per cent. globulin and 34 to 54 per cent. globulin, respectively. By the same technique a similar quantity of the same lot of serum was fractioned by salting out at 38 and 42 per cent. saturation, the residual globulin in each case being separated from the albumin in the filtrate by precipitation at 54 per cent. saturation ammonium sulphate.

*Relative Potency of the Globulins Obtained by Salting Out with Progressive Amounts of Ammonium Sulphate.*

Amount of culture II.	Amount of serum fractions.	Controls. Culture alone.	Globulins precipitated by ammonium sulphate saturation within the limits						Original serum.
			0-34%	34-54%	0-38%	38-54%	0-42%	42-54%	
0.01 c.c.	0.2 c.c.	.....	S.	D. 20	S.	D. 22	S.	D. 18	S.
0.001 c.c.	0.2 c.c.	.....	S.	D. 72	S.	D. 24	S.	D. 24	S.
0.0001 c.c.	0.2 c.c.	D. 36	S.	D. 18	S.	D. 36	S.	D. 36	S.
0.00001 c.c.	0.2 c.c.	D. 36	S.	S.	S.	D. 36	S.	D. 36	S.
0.000001 c.c.	0.2 c.c.	D. 36	S.	S.	S.	D. 36	S.	D. 36	S.

*Agglutination with Various Globulin Fractions.*

Serum II, lot 3 .....	++
0-34 per cent. globulin .....	++
34-54 per cent. globulin .....	±
0-38 per cent. globulin .....	++
38-54 per cent. globulin .....	—
0-42 per cent. globulin .....	++
42-54 per cent. globulin .....	—

Experiments 1 to 8 show that the immune bodies occurring in antipneumococcus sera I and II are associated or combined with the globulins, but are not confined solely to any one of the globulin

fractions obtained by the various methods of separation employed. Since certain of the experiments seemed to indicate that the major portion of the antibacterial substances are crowded toward the true or euglobulin end of the protein spectrum, an attempt was made in experiment 9 to determine, by fractional salting out with progressive amounts of ammonium sulphate, a point where the zones of globulin and antibody precipitation might coincide and the total potency of the serum be conserved with a minimum of serum globulin. Experiment 9 indicates that this zone of joint precipitation of total antibody with minimal globulin is reached at about 38 per cent. saturation with ammonium sulphate. At this point a fraction of the serum globulin is precipitated which is as active in antibacterial potency as the original serum, while the residual globulin is inactive and apparently unessential.

#### DISCUSSION.

The chemistry of the proteins of immune serum and their relation to various antibodies have been studied thoroughly in the antitoxic sera. Numerous investigators have shown that diphtheria and tetanus antitoxin are precipitated with the serum globulins. More exact study of these globulins has demonstrated that they may be further subdivided according to their solubility and precipitation by certain chemical reagents. Seng showed that the globulins of immune serum, and according to Marcus<sup>8</sup> those of normal serum as well, are of two kinds: an insoluble globulin precipitated by acetic acid, carbon dioxide, dilution with water, or dialysis; and a soluble globulin unaffected by these reagents but precipitated by the neutral salts of ammonium and magnesium sulphate. Seng showed, and many investigators have since confirmed, the association of antitoxin with the soluble globulin. Fractional precipitation with the neutral salts of the heavy metals yields a similar, though not altogether comparable, separation of the globulins into a soluble and insoluble fraction, the so called euglobulin and pseudoglobulin. It is with the latter of these, the pseudoglobulin, that antitoxin is combined. By the application of this principle Gibson and Banzhaf have devised an efficient and economic method for the concentration of diphtheria antitoxin.

<sup>8</sup> Marcus, E., *Ztschr. f. physiol. Chem.*, 1899, xxviii, 559.

It is now generally accepted that the antibodies occurring in immune serum are protein in character and are not readily dissociated from the protein of the serum in which they occur. Pröscher's<sup>9</sup> attempt to produce a non-protein antitoxin by digestion of antidiphtheria serum with trypsin has not been confirmed. Banzhaf<sup>10</sup> and Mellanby found that the ratio of protein digestion and antibody destruction are approximately the same. Seng, Joachim,<sup>11</sup> Atkinson,<sup>12</sup> Ledingham,<sup>13</sup> and Banzhaf found that certain quantitative changes occur in the serum proteins of animals immunized to bacterial toxins, and that the increase in the globulin content bears a marked relation to the increase in antitoxic potency of the serum. Landsteiner<sup>14</sup> has shown that the antitryptic action of normal serum is associated with the albumin fraction, and Opie and Barker<sup>15</sup> have found antileucoprotease in the same fraction.

The distribution of immune bodies in the blood serum of different animal species varies. Pick<sup>16</sup> found that in immune horse serum diphtheria antitoxin was associated with the pseudoglobulin, while in goat serum it is present in the euglobulin. The lysins and agglutinins of cholera occur in the euglobulin fraction of the serum of immunized goats and horses. Typhoid agglutinins in the immune serum of the horse are found in the pseudoglobulin, while in goats, rabbits, and guinea pigs they occur only in the euglobulin. Hartley<sup>17</sup> has recently shown that the immune bodies of antirinderpest serum are present in the euglobulin fraction of bovine serum.

The present work on the distribution of the immune bodies of antipneumococcus serum is confined to a study of their occurrence in the blood serum of horses immunized to types I and II of the pneumococcus. The sera were produced by repeated intravenous injections of these organisms, beginning with dead bacteria and

<sup>9</sup> Pröscher, *München. med. Wchnschr.*, 1902, xlix, 1176.

<sup>10</sup> Banzhaf, E. J., *Bull. Johns Hopkins Hosp.*, 1911, xxii, 106.

<sup>11</sup> Joachim, J., *Arch. f. d. ges. Physiol.*, 1903, xciii, 558.

<sup>12</sup> Atkinson, J. P., *Jour. Exper. Med.*, 1900-01, v, 67.

<sup>13</sup> Ledingham, J. C. G., *Jour. Hyg.*, 1907, vii, 65.

<sup>14</sup> Landsteiner, K., *Centralbl. f. Bakteriol., 1te Abt., Orig.*, 1900, xxvii, 357.

<sup>15</sup> Opie, E. L., and Barker, B. I., *Jour. Exper. Med.*, 1907, ix, 207.

<sup>16</sup> Pick, E. P., *Beitr. z. chem. Phys. u. Path.*, 1902, i, 351.

<sup>17</sup> Hartley, P., *Memoirs of the Department of Agriculture, India*, 1913-14, i,

progressively increasing the doses, until the animals were able to tolerate the bacterial residue of a liter of live virulent pneumococcus injected directly into the blood stream. The serum was obtained from horses which had been immunized over periods of one to two years. The method employed in obtaining antipneumococcus serum differs entirely from that used in the production of antitoxins. In the former, the immunizing response is provoked by a live virulent organism, while in the latter the stimulus is in the nature of a bacteria-free toxin. The immunity mechanism involved in the elaboration of specific antipneumococcus substances may or may not differ physiologically from that concerned in the production of antitoxins, but the types of antibody evoked in each instance are distinct, and their distribution in the serum globulins is different.

#### SUMMARY.

The immune bodies of antipneumococcus serum are completely precipitated by 38 to 42 per cent. saturation with ammonium sulphate.

They are incompletely precipitated by (a) ammonium sulphate in less than 38 per cent. saturation, (b) saturation with sodium chloride, (c) dilution and saturation with carbon dioxide, (d) removal of crystalloids by dialysis.

The immune bodies of antipneumococcus serum are, therefore, associated or combined with that fraction of the globulins precipitated by 38 to 42 per cent. saturation with ammonium sulphate. The immune body fraction does not correspond exactly with the ordinary euglobulin (one-third saturation with ammonium sulphate or complete saturation with sodium chloride) or with the insoluble globulins precipitated by carbon dioxide or dialysis. These fractions carry with them only a part of the immune bodies.

Neither the albumin nor that fraction of the globulin not precipitated by 38 to 42 per cent. saturation of ammonium sulphate contain any of the demonstrable antibodies.

The most promising method for the practical purification of the immune bodies occurring in antipneumococcus serum appears to be precipitation by 38 to 42 per cent. saturation with ammonium sulphate.

## THE TYPES OF PNEUMOCOCCI IN TUBERCULOUS SPUTUM.\*

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The success of Cole<sup>1</sup> and his associates<sup>2</sup> in establishing four groups among the pneumococci affords a new basis for the study of the transmissibility and pathology of acute lobar pneumonia. The most important result of this work is, perhaps, its bearing on the epidemiology of the disease. It has been generally accepted that the pneumococci of the normal mouth may, under favorable conditions, become implanted in the lung and are there able to set up the condition known as lobar pneumonia. The work of Rosenow<sup>3</sup> has been considered as favoring this theory by supposing that the pneumococci of the mouth, under changed environmental conditions, undergo a mutation into a virulent type. On the other hand, the work indicating that different types of pneumococci may operate in producing pneumonia, raises the question as to whether pneumonia may not be a transmissible or epidemic disease caused by distinct, virulent types of this organism, passed from individual to individual. To prove the first theory, it must be shown either that the organism of acute pneumonia and that ordinarily present in the healthy mouth are the same, or, postulating a mutation, that the comparatively avirulent mouth strain, as a result of the changed lung condition, assumes the characteristics of one of the virulent groups associated with the acute disease.

It was with this problem in mind that the present work was undertaken. In pulmonary tuberculosis and the other lung conditions

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<sup>1</sup> Cole, R., *Pneumococcus Infection and Lobar Pneumonia*, *Arch. Int. Med.*, 1914, xiv, 56.

<sup>2</sup> Dochez, A. R., and Gillespie, L. J., *A Biologic Classification of Pneumococci by Means of Immunity Reactions*, *Jour. Am. Med. Assn.*, 1913, lxi, 727.

<sup>3</sup> Rosenow, E. C., *Transmutations within the Streptococcus-Pneumococcus Group*, *Jour. Infect. Dis.*, 1914, xiv, 1.

represented in the present series of cases there is to be found, perhaps, the nearest approach to the pathological condition found in the pneumonic lung. If the changed condition of the lung tissue is to be regarded as the important factor in the transformation of the pneumococci ordinarily present in the mouth into the virulent organisms of acute pneumonia, it might be reasonably expected that the similarly changed conditions in tuberculosis would bring about the same result. If, in a study of such cases, it were to be found that the virulent groups I and II predominated, this would be strong evidence that the access to pathological lung tissue called forth a definite change in the biological characteristics of the pneumococcus.

In the present series fifty cases were studied with a view to determining the type of pneumococcus present in the sputum. These cases were, with one exception, patients at the New York State Hospital for Incipient Tuberculosis. The one exception, case 32, upon examination proved to be acute bronchial asthma. Of the remaining forty-nine, forty-three were cases of frank pulmonary tuberculosis, five were diagnosed as bronchiectasis (probably non-tuberculous), and one was a case of chronic asthma in which the diagnosis of tuberculosis had not been definitely made. The fact that all patients had received sanatorium treatment for varying lengths of time might conceivably have had some influence on the bacterial flora of the mouth or sputum.

In the selection of patients the more advanced cases of tuberculosis were purposely chosen. As judged by the Turban scale, the Gaffky count, and the general physical findings in the monthly examinations, the forty-three cases were distributed as follows: stage 1, three; stage 2, nineteen; and stage 3, twenty-one. Of these, eighteen were cavity cases, and eleven were subject to recurrent hemoptysis. Whenever possible, these latter cases were examined during the height of the hemorrhage. The five cases of bronchiectasis were of long duration, with copious expectoration, and all were probably non-tuberculous. One of the two cases of asthma was of the chronic type, complicated with some symptoms which were suspicious of tuberculosis. The other was acute bronchial asthma. Only three gave a history of a previous pneumonia, two of which were recent. It is interesting to note that from case 43, with a

history of pneumonia in January, 1914, an organism of group I was isolated.

The following routine procedure was adopted. The first sample examined was that of the unwashed sputum. This consisted of deep morning sputum, expectorated directly into a sterile Petri dish. This was carried to the laboratory and carried through as quickly as possible, as follows:

A small piece of sputum, about the size of a pea, was rubbed up in a sterile mortar with a little nutrient bouillon. 0.5 to 1 c.c. of this mixture was injected into the abdominal cavity of a white mouse weighing 15 to 20 gm. Upon the death of the animal, which usually occurred inside of forty-eight hours, an aseptic autopsy was performed and cultures were made from the peritoneal exudate and heart's blood on plain blood agar and plain beef broth (reaction +0.3 to +0.6 per cent. to phenolphthalein). In practically all cases, a pure culture of the infecting organism was obtained. In addition to the cultures, the spleens of all animals were saved and preserved by desiccation. In this way a stock culture was retained in case of an accident to the broth or agar culture. Capsule and Gram stains were done on the peritoneal exudates and heart's blood of all animals. The washed specimen was treated in the same manner, except that it was first thoroughly washed through several changes of sterile saline, according to the Kitasato method as modified by the Saranac Lake Laboratory.

All organisms, when finally isolated in pure culture, were tested for their power to ferment inulin, to coagulate litmus milk, and for their solubility in bile. If these three tests were positive, and the organism showed a capsule, agglutination tests with sera I and II were done, according to the technique used at The Rockefeller Institute Hospital. Finally, pathogenicity and protection tests were carried out with each organism. However, before this was done, the virulence of the strains was increased by mouse passage. This is necessary with the majority of sputum strains in order to insure reasonably uniform results in the protection experiments. The protection was carried out in accordance with the routine technique used at The Rockefeller Institute Hospital, and was as follows:

Dilutions, from 0.1 to 0.00001, of a twenty-four hour plain broth culture, were made, so that 0.5 c.c. of dilution contained the required amount of culture. The protective sera were also diluted so that one protective unit (0.2 c.c.) was contained in 0.5 c.c. All injections were made intraperitoneally. Three series of mice were treated as follows: series 1 received varying dilutions of the broth culture, called the pathogenicity control; series 2, the same dilutions of culture plus one unit of serum I; series 3, the same dilutions of culture plus one unit of serum II. The animals were kept under observation for a period of five days. On account of its greater delicacy, the protection test is considered more definite than the agglutination.

Table I presents the findings together with a brief description of each case.



TABLE I.

Case No.	Stage, Turban.	Gaffky sputum count.	Description of case.	Prognosis.	Pneumococcus in sputum unwashed.	Pneumococcus in sputum washed.	Virulence.	Remarks.
1	III	IV	Active lesion; recent hemorrhages	Unfavorable	0	0	.....	2 specimens examined. Patient hemorrhaging at time of collection.
2	III	IV	Progressive; toxic; hydrothorax	Died	IV	IV	0.1 D. 96 0.01 D. 96 0.001 D. 120 0.0001 S. 120 0.00001 D. 96	Patient died 1 mo. after examination.
3	III	VI	Large cavity; no hemoptysis; lesion stationary at present	No improvement to be expected	0	0	.....	2 specimens examined.
4	II	IV	Active lesion; toxic	Not good	I	I	0.1 D. 24 0.01 D. 24 0.001 D. 24 0.0001 D. 48 0.00001 D. 48	.....
5	II	V	Recurrent hemoptysis; no cavity; chronic type	Unfavorable	IV	IV	0.1 D. 24 0.01 D. 24 0.001 D. 24 0.0001 D. 48 0.00001 D. 48	.....
6	III	IV	Large cavity; lesion progressive	Bad	0	0	.....	2 specimens examined.
7	III	V	Small cavity (old); lesion stationary	Fair	0	0	.....	2 specimens examined.
8	II	Neg.	Arrested hemorrhage case	Favorable	0	0	.....	Friedländer's bacillus recovered.
9	....	Neg.	Bronchiectasis	Unfavorable	0	0	.....	.....
10	II	Neg.	Hemorrhage; bronchiectasis	Favorable	IV	IV	0.1 D. 24 0.01 D. 24 0.001 D. 24 0.0001 D. 72 0.00001 S. 120	.....
11	III	VIII	Cavity; active	Unfavorable	0	0	.....	2 specimens examined.
12	II	Neg.	Pneumothorax; lesion quiescent	Favorable	0	0	.....	.....

D. = died; S. = survived. The figures represent the number of hours before the death of the animal.

Inulin, bile, and litmus milk were positive on all except strain 47, which was insoluble in bile.

TABLE I.—Continued.

Case No.	Stage, Turban.	Gaffky sputum count.	Description of case.	Prognosis.	Pneumococcus in sputum unwashed.	Pneumococcus in sputum washed.	Virulence.	Remarks.
13	....	Neg.	Bronchiectasis; history of previous pneumonia	Unfavorable	III	III	0.1 D. 24 0.01 D. 24 0.001 D. 24 0.0001 D. 36 0.00001 D. 36	2 specimens examined.
14	III	III	Old cavity; no hemoptysis	Bad	0	0	.....	.....
15	....	Neg.	Bronchiectasis; lesion improving	Fair	0	0	.....	2 specimens examined.
16	III	VIII	Large cavity; progressive	Very bad	0	0	.....	.....
17	II	Neg.	Beginning tissue destruction; progressive case	Bad	0	0	.....	.....
18	III	VI	Recent hemorrhage; progressive	Very unfavorable	0	0	.....	2 specimens examined.
19	II	IV	Active lesion	Favorable	IV	IV	0.1 D. 24 0.01 D. 24 0.001 D. 24 0.0001 D. 24 0.00001 D. 24	.....
20	II	VII	Active; progressive	Unfavorable	0	0	.....	3 specimens examined.
21	II	VI	Chronic	Unfavorable	IV	IV	0.1 D. 24 0.01 D. 24 0.001 D. 24 0.0001 D. 72 0.00001 S. 120	.....
22	II	IV	Arrested hemorrhage case	Favorable	0	0	.....	.....
23	II	III	Recurrent hemoptysis	Fair	0	0	.....	.....
24	II	IV	Hemorrhage; active lesion	Favorable	IV	IV	0.1 D. 24 0.01 S. 120 0.001 S. 120 0.0001 S. 120 0.00001 S. 120	Patient hemorrhaging at time of examination.
25	II +	VI	Cavity; hemorrhage	Unfavorable	0	0	.....	2 specimens examined, 2 and 4 dys. after hemorrhage.
26	II	IV	Miliary deposits; hemorrhage	Favorable	0	0	.....	2 specimens examined, 1 and 3 dys. after slight hemorrhage.

TABLE I.—Continued.

Case No.	Stage, Turban.	Gaffky sputum count.	Description of case.	Prognosis.	Pneumococcus in sputum unwashed.	Pneumococcus in sputum washed.	Virulence.	Remarks.
27	II	VIII	Active lesion; hemorrhage; pneumonia 18 yrs. ago	Favorable	0	0	.....	.....
28	I	VI	Active lesion	Favorable	0	0	.....	.....
29	III	IV	Lesion stationary; occasional hemorrhage	Unfavorable	0	0	.....	.....
30	III	IX	Cavity; progressive	Unfavorable	IV	IV	0.1 D. 24 0.01 D. 48 0.001 D. 48 0.0001 D. 48 0.00001 D. 72	.....
31	III	IV	Cavity; progressive	Unfavorable	IV	IV	0.1 D. 24 0.01 D. 24 0.001 D. 48	.....
32	....	Neg.	Bronchial asthma; non-tuberculous	Improvement marked	III	Not done	0.1 D. 18 0.01 D. 18 0.001 D. 18 0.0001 D. 36 0.00001 D. 36	.....
33	III	III	Base cavity; progressive	Unfavorable	0	0	.....	.....
34	III	III	Progressive	Very bad	0	0	.....	Gaffky count has been higher.
35	III	V	Large cavity; progressive	Very bad	IV	IV	0.1 D. 24 0.01 D. 24 0.001 D. 48 0.0001 S. 120	Sputum always blood-streaked.
36	III	IV	Cavity; progressive	Bad	0	0	.....	.....
37	III	IV	Cavity; progressive	Bad	IV	IV	0.1 D. 24 0.01 D. 24 0.001 D. 72 0.0001 D. 96 0.00001 S. 120	Presumptive evidence of intestinal tuberculosis.
38	III	V	Large cavity; progressive	Bad	0	0	.....	.....
39	II	Neg.	Recent large hemorrhage; lesion slowly progressive	Unfavorable	0	0	.....	.....
40	III	III	Cavity; progressive	Unfavorable	0	0	.....	.....
41	III	II	Old cavity; chronic case	Unfavorable	0	0	.....	.....

TABLE I.—*Concluded.*

Case No.	Stage, Turban.	Gaffky sputum count.	Description of case.	Prognosis.	Pneumo-coccus in sputum unwashed.	Pneumo-coccus in sputum washed.	Virulence.		Remarks.
42	....	Neg.	Cavity quiescent; possibly bronchiectasis	Very favorable	IV	1st specimen III 2d specimen IV	0.1 D. 24 0.01 D. 24 0.001 D. 48		Virulence done on unwashed strain.
43	II	VI	Chronic case, 15 yrs. duration; pneumonia, Jan., 1914	Favorable	I	I	0.1 D. 24 0.01 D. 24 0.001 D. 24 0.0001 D. 24 0.00001 D. 24		On protection against serum I, all mice died on 5th dy. Agglutination typical; immediate.
44	II	III	Lesion stationary	Favorable	o	o	.....		.....
45	I	III	Occasional hemorrhage; condition good	Favorable	III	IV	0.1 D. 24 0.01 D. 24 0.001 D. 24 0.0001 D. 24 0.00001 D. 48		Virulence is recorded for unwashed strain. Virulence not done on washed strain.
46	II	V	Condition good	Good	o	o	.....		.....
47	II	III	Lesion stationary; improving	Good	IV	IV	0.1 D. 24 0.01 D. 24 0.001 D. 72 0.0001 D. 48 0.00001 D. 24		This strain not soluble in bile.
48	....	Neg.	Bronchial asthma; tuberculosis questionable	Good	IV	Not done	0.1 D. 24 0.01 D. 24 0.001 D. 24 0.0001 D. 24 0.00001 D. 24		Washed specimen not done. Patient had gone home.
49	III	III	Old cavity; slowly progressive	Unfavorable	IV	IV	0.1 D. 24 0.01 D. 24 0.001 D. 24 0.0001 D. 24 0.00001 S. 120		.....
50	....	Neg.	Bronchiectasis	Favorable	IV	IV	0.1 D. 24 0.01 D. 24 0.001 D. 24 0.0001 D. 72 0.00001 D. 96		.....

In the fifty cases tabulated pneumococci were isolated in twenty only, or 40 per cent. With the exception of two cases in which no washed specimen was examined, there were only two differences in the findings on the washed and unwashed specimens. The percentage of positive findings is low compared with the figures given for pneumococci in normal mouths by Longcope and Fox,<sup>4</sup> Buerger,<sup>5</sup> and Wadsworth.<sup>6</sup> This may be due to the favorable influence of sanatorium treatment. On the other hand, the percentage figure is considerably higher than that ordinarily reported for this type of organism as a secondary invader in pulmonary tuberculosis.<sup>7</sup> There are two possible explanations of the higher figure in this instance; first, the application of the more exact technique, and, second, the fact that, as previously mentioned, only cases were selected for examination which showed a progressive lung condition.

Of the forty-three cases of tuberculosis, pneumococci were found in fifteen (34.9 per cent.). The positive cases included six out of the eighteen with definite cavity formation, and four out of the eleven hemorrhage cases. There seems to be no definite correlation between the type of case and the presence of pneumococci in the sputa. Positive results were obtained in three out of five cases of bronchiectasis and in both cases of asthma.

By classifying the pneumococci isolated according to groups, it will be noted that, of the twenty positive cases, group IV organisms were found in fifteen (75 per cent.), group III in three (15 per cent.), and group I in two (10 per cent.), when case 43 is included. No organisms of group II were isolated. Dochez and Avery<sup>8</sup> have

<sup>4</sup> Longcope, W. T., and Fox, W. W., A Comparative Study of Pneumococci and Streptococci from the Mouths of Healthy Individuals and from Pathological Conditions, Report of the Medical Commission for the Investigation of Acute Respiratory Diseases, Department of Health of the City of New York, *Jour. Exper. Med.*, 1905, vii, 430.

<sup>5</sup> Buerger, L., Studies of the Pneumococcus and Allied Organisms with Reference to Their Occurrence in the Human Mouth, Report of the Medical Commission for the Investigation of Acute Respiratory Diseases, Department of Health of the City of New York, *Jour. Exper. Med.*, 1905, vii, 497.

<sup>6</sup> Wadsworth, A., Experimental Studies on the Etiology of Acute Pneumonitis, *Am. Jour. Med. Sc.*, 1904, cxxvii, 851.

<sup>7</sup> Avery, O. T., and Lyall, H. W., Concerning Secondary Infection in Pulmonary Tuberculosis, *Jour. Med. Research*, 1913, xxviii, 111.

<sup>8</sup> Dochez, A. R., and Avery, O. T., Varieties of Pneumococcus and Their Relation to Lobar Pneumonia, *Jour. Exper. Med.*, 1915, xxi, 114.

shown that the organisms usually present in the mouths of normal persons belong in group IV, while pneumococci of the so called fixed types, especially those of groups I and II, occur with great infrequency, and only under such conditions as apparently to justify considering persons who harbor them as carriers of infection. The study of this small number of cases of tuberculosis indicates that the type of pneumococci present is usually that found in the normal mouth, and that the fixed types present in the majority of cases of acute lobar pneumonia occur only infrequently. Further study will be required to show whether the fixed types occur more commonly in the mouth and sputum of patients with tuberculosis than they do in the mouths of normal persons. It must be remembered that the organisms placed in group III are so classified on certain morphological and cultural characters. Whether all the organisms placed in this group are identical from the standpoint of infection and immunity is not yet certain.

It is of significance that, among the twenty organisms studied, only two belonged in groups I and II. One of the patients harboring such an organism had had an attack of pneumonia six months previously. While the evidence obtained does not exclude the possibility that transformation or mutation of type may occur in cases with pathological lung lesions, the occasional occurrence of specialized types in these cases is probably to be explained on other grounds.

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FURTHER INVESTIGATIONS ON THE MODE OF  
ACTION OF SUBSTANCES INHIBITING  
TUMOR GROWTH AND ON IMMUNI-  
ZATION AGAINST THESE  
SUBSTANCES.\*

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The results which we obtained in our previous investigations on the mode of action of substances inhibiting tumor growth<sup>1</sup> were based partly on statistical methods; we therefore hesitated in our previous communications to pronounce our results as definite. The importance of these results, however, seemed to us to call for further experiments which should test the validity of our conclusions and extend the experimental basis on which they rested. In order to confirm these experiments we used a large number of mice, 604 of which lived to the end of the experiments and are included in our report.

THE SPECIFICITY OF THE IMMUNIZING ACTION OF INJECTIONS OF  
HIRUDIN AND COLLOIDAL COPPER.

In our previous paper<sup>2</sup> we concluded from the results of experiments carried out with the first method that the immunity against the influence of colloidal copper and hirudin in inhibiting tumor growth conferred through several injections of these substances, is, in the main, specific; that injections of colloidal copper immunize principally against the effects of colloidal copper and not, or only to a slight extent, against those of hirudin, and that hirudin immunizes principally against the effects of hirudin.

It was much to be desired that these conclusions should be further tested by means of the more exact second method. We made, there-

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<sup>1</sup> Fleisher, M. S., Vera, M., and Loeb, L., *Jour. Exper. Med.*, 1914, xx, 522.

<sup>2</sup> Fleisher, Vera, and Loeb, *loc. cit.*

fore, two additional experiments, the results of which are given in table I in horizontal columns 3 and 4.

TABLE I.

	No. of mice.	$\alpha$	$\beta$	$\gamma$	$\delta$	$\alpha$	$c$	$d$	$f$	Horizontal column.
E-1 Controls	22	—	—	80%	—	—	4	10	8	1
E-1 Copper controls: 9th-13th dy.	51	66%	55%	58%	60%	8	14	18	11	
E-1 Immunized: 2d-6th dy.	52	98%	87%	88%	91%	8	12	19	13	
Copper: 9th-13th dy.	52	98%	87%	88%	91%	118%	84%	77%	74%	
E-2 Controls	18	138%	144%	136%	139%	3	2	7	6	2
E-2 Copper controls: 9th-13th dy.	64	87%	81%	78%	83%	7	9	21	27	
E-2 Copper series: transplanted 9th-13th dy.	70	138%	135%	134%	136%	10	20	24	16	
	70	138%	135%	134%	136%	121%	117%	147%	150%	
F Controls	31	158%	158%	167%	161%	7	13	7	4	3
F Hirudin: 2d-6th dy.	72	157%	149%	152%	153%	11	19	26	16	
Hirudin: 9th-13th dy.	72	157%	149%	152%	153%	173%	148%	143%	145%	
F Hirudin: 2d-6th dy.	72	123%	117%	122%	120%	11	24	24	13	
Copper: 9th-13th dy.	72	123%	117%	122%	120%	163%	108%	112%	105%	4
G Controls	28	141%	149%	150%	147%	—	6	8	14	
G Copper: 2d-6th dy.	61	76%	75%	73%	74%	—	8	23	30	
Hirudin: 9th-13th dy.	61	76%	75%	73%	74%	—	59%	98%	62%	
G Copper: 2d-6th dy.	63	150%	136%	147%	144%	—	6	22	35	
Copper: 9th-13th dy.	63	150%	136%	147%	144%	—	175%	134%	131%	

$\alpha$  Percentage of increase on comparing the sum of the original weight of all tumors (calculated) with the sum of the end weight (weighed).

$\beta$  Multiply the average percentage of increase of each class ( $\alpha$ ,  $c$ ,  $d$ ,  $f$ ) by the number of mice in that class, add the figures thus obtained, and divide the sum by the total number of mice used in that series.

$\gamma$  Average percentage of increase of the various classes; add the percentage increases in each class and divide by the number of classes.



δ Average of the three preceding averages.

In the tables the series in each column which have the same mark, for instance A1 and B1, represent experiments done under approximately the same conditions and at the same time.

The figures above and to the left of the main figures indicate the number of mice used in each class.

In the experiment recorded in column 3, 175 mice lived until the end of the experiment; 31 served as controls; 144 mice received from the second to the sixth day a daily injection of hirudin, each animal thus receiving four preliminary (preparatory) injections. Of these 144 mice, one-half received from the ninth to the thirteenth day injections of hirudin, while the second half received colloidal copper. The same doses of these substances were used as in our previous experiments.<sup>3</sup> According to the weight of the mouse tumors on the ninth day, we divided them into the same four lots (a, c, d, f) as in the previous experiments. Such a division is necessary because the rapidity of growth of a tumor in a given period is, among other factors, as a rule, a function of its weight. In these experiments the difference in the various classes happens to be slight. The increase in weight in the different classes from the ninth to the thirteenth day is given in the vertical columns. In the vertical columns,  $\alpha$ ,  $\beta$ ,  $\gamma$ ,  $\delta$ , the average of the increase in weight of the tumors of all the mice treated in the same manner is given, calculated according to the four different procedures which have been described in our previous paper.<sup>4</sup> We find that the mice which had been treated with injections of hirudin from the second to the sixth day were only slightly affected by the second set of injections of hirudin. The figures showing the percentage increase in weight from the ninth to the thirteenth day, during which period the animals received injections of hirudin, were only slightly less than those of the controls; while, on the other hand, the mice which received during this same period injections of colloidal copper were noticeably inhibited in their tumor growth. We may therefore conclude that while the preliminary injections of hirudin immunized the animals almost completely, or at least very markedly, against the effect of later injections of hirudin, they did not exert such an effect towards

<sup>3</sup> Fleisher, Vera, and Loeb, *loc. cit.*

<sup>4</sup> Fleisher, Vera, and Loeb, *loc. cit.*

later injections of colloidal copper, in so far as the immunizing effect of preliminary injections of hirudin is a specific one. This conclusion is confirmed by the result of the experiment in horizontal column 4. 124 animals received from the second to the sixth day after inoculation daily intravenous injections of colloidal copper; 63 of these mice received again colloidal copper from the ninth to the thirteenth day; while 61 mice received hirudin from the ninth to the thirteenth day. In the first lot, immunized against the action of colloidal copper, the colloidal copper exerted no, or only a very weak inhibiting action on the tumors, which grew therefore almost as well as in the controls; while in the second lot, which was again immunized against colloidal copper, but not against hirudin, injections of hirudin exerted a marked inhibiting action on tumor growth. This experiment at the same time proves that the hirudin used in the experiment recorded in horizontal column 3 would have been sufficiently strong to exert an inhibiting effect in the latter experiment, provided that the mice had not been immunized against the inhibiting action of hirudin. We may, therefore, on the basis of these, and of our previously recorded experiments, conclude that the immunizing action of preliminary injections of hirudin and colloidal copper is specific.

THE IMMUNIZATION OF TUMOR CELLS AGAINST THE EFFECT OF COLLOIDAL COPPER AND THE TRANSMISSION OF THIS IMMUNITY TO SUCCEEDING CELL GENERATIONS.

Further experiments were carried out in order to test and, if possible, to confirm our previous results, which indicated that the immunity produced against substances inhibiting tumor growth resides partly in the tumor cells and is transmitted to succeeding generations of tumor cells. In horizontal column 2, table I, an additional experiment of this kind is recorded. 152 animals lived to the end of the experiment; 18 were controls, not injected animals; all the others received from the ninth to the thirteenth day injections of colloidal copper; 70 of these 134 mice had been inoculated with pieces from tumors grown in mice which had received injections of colloidal copper from the second to the sixth day and from the ninth to the thirteenth day. An immunity had therefore presumably been

established in the tumor cells against the effect of colloidal copper. That this is the case is shown in horizontal column 2. In the last lot of 70 mice, the tumors grew approximately as well as in the controls, despite the injections of colloidal copper which these 70 mice received, while in the 64 mice with non-immunized tumors, the tumor growth was inhibited to the usual extent through the injections of colloidal copper given from the ninth to the thirteenth day. In addition we carried out another experiment in which is compared the effect of colloidal copper on tumors in 52 animals which had been immunized against the effect of colloidal copper through preceding injections with this substance, given from the second to the sixth day, and in 51 animals which had not received a series of preliminary injections. We see that in the immunized animals the tumors grew about as well as in the non-injected controls, while in the non-immunized animals the tumor growth was inhibited through colloidal copper. These results again confirm our previous conclusions.

#### THE INEFFECTICACY OF INJECTIONS OF COLLOIDAL COPPER AND OF HIRUDIN ON VERY YOUNG TUMORS.

As we have observed previously, the weight of a tumor on the ninth day after transplantation usually enters as a factor into the set of conditions which determine the increase in weight of the tumors in the following four days. In order to rule out a possible influence of this factor on the results of our experiments, we had to compare on the ninth day the average weight of the various lots of tumors, arranged in the various classes. Tumors of smaller weight usually show in the following period from the ninth to the thirteenth day a greater increase in weight than tumors of larger weight. We must examine whether the differences in the weight of the various lots correspond and can be held responsible for the differences observed in weight increase from the ninth to the thirteenth day in immunized and non-immunized mice. Such a table should also give evidence of a possible inhibiting effect of intravenous injections of colloidal copper or of hirudin given from the second to the sixth day. If such an inhibiting effect should exist, the average weight of tumors on the ninth day in mice which received these preliminary

TABLE II.  
*Weight of Various Classes of Tumors at Nine Days.*

Column 1. Normal uninjected controls.				Column 2. Mice injected with colloidal copper 9th-13th dy.				Column 3. Mice injected with colloidal copper 2d-6th and 9th-13th dys.				Column 4. Mice inoculated with tumor from mice injected with colloidal copper. Injected with colloidal copper 9th-13th dy.			
a	c	d	f	a	c	d	f	a	c	d	f	a	c	d	f
H				A-1	5	9	10					A-1	18	12	21
D	8	35	36	A-2	1286	176	58	A-2				A-2	247	173	51
B-1	240	158	92		6	5	23		5	7	6				
I	6	6	6	B-2	283	198	68	B-1	270	162	87				
350	271	180	91	17	30	8	4	12	26	14	8				
B-2				B-2	281	177	106		291	186	92				
10	6	5	18	10	14	12	13					B-2	22	18	13
525	291	137	72	C-1	297	156	92						421	160	60
3	12	3	—	14	15	14	15	C-1	4	16	16		280		
C-2	276	190		439	293	162	87		447	180	84				
		15	35	8	20	15	23								
E-1	—	143	68	E-1	284	156	73						22	22	18
	4	10	8	8	14	18	11	E-1	8	19	13		411	159	101
E-2	308	179	96	434	264	176	94		380	162	92				
3	2	7	6	E-2	9	21	27					E-2	20	24	16
426	285	166	73	7	277	164	85					10	202	167	94
				413								426			
Controls:				F Hirudin-copper or	G Copper-			F Hirudin-	G Copper-						
F Specimen 1				F				F							
7	13	7	4	11	24	24	13	11	19	26	16				
431	294	180	110	437	294	185	103	415	293	175	104				
G Specimen 2	6	8	14	G	6	22	35	G	8	23	30				
—	267	153	94	—	263	149	95		255	147	94				

The letters and numbers of each group in table II correspond to the designations in table I of this paper and table I of our previous paper.<sup>5</sup> By comparing this table with the two other tables, it will therefore be possible to determine the increase in weight from the ninth to the thirteenth day of the tumors of the various classes of mice.

injections should be less. We notice that the animals which received injections of colloidal copper or of hirudin from the second to the sixth day had on the ninth day tumors of approximately the same weight as mice without such injections. In column 3, table II, is given the average weight of the tumors of the various lots of mice in classes, a, c, d, f, according to the weight limits fixed in our previous paper. We see that there is no noticeable difference in the average weight of the different classes of mice, if we compare columns 3 and 2. In the latter is the weight of the tumors of mice which had not received the preliminary injections. In the last two horizontal columns (F and G) we can compare the weight on the ninth day of tumors of non-injected mice (vertical column 1) with those having received hirudin (F vertical column, Nos. 2 and 3) and colloidal copper (G vertical column, Nos. 2 and 3) from the second to the sixth day. We see that on the whole there is no marked difference in the weight of the tumors of the different lots of mice.

In vertical column 4 we find a few classes of tumors with somewhat smaller weight; namely, c and f in horizontal column A<sup>1</sup>, and in group F in horizontal column B<sup>2</sup>. All others are approximately normal. If we analyze the figures indicating the increase in weight of the tumors from the ninth to the thirteenth day of mice inoculated with immunized tumor material, consulting for this purpose the increase of weight of these tumors given in table I of our previous paper<sup>6</sup> and in table I of this paper, we see that our conclusion as to the lack of inhibition in the growth of tumors of these lots of mice is not dependent on these few classes, but is noticeable in other classes as well. We may therefore conclude that the immunization which we observed in mice having received a preliminary set of injections, either from the second to the sixth day after inoculation, or in the previous generation of mice, is real and is not due merely to the difference in weight of the tumors on the ninth day. We may furthermore conclude that injections of colloidal copper or hirudin at an early stage (from the second to the sixth day) after transplantation do not inhibit tumor growth to a noticeable extent.

<sup>6</sup> Fleisher, Vera, and Loeb, *loc. cit.*

THE EFFECT OF A COMBINATION OF HIRUDIN AND COLLOIDAL COPPER  
ON TUMOR GROWTH.

In our previous paper we reported that combinations of colloidal copper and hirudin and of colloidal copper and nucleoproteid were much more effective than either of these substances alone, and that these combinations not only caused an inhibition of the tumor growth but also retrogression of a considerable number of tumors; we also found that the combination of colloidal copper and hirudin was more toxic than either substance given alone. In order to confirm this result we carried out an additional experiment of which table III gives the results.

TABLE III.

Substance injected.	Dose.	Number of tumors and mortality.	Result.
Combination of hirudin and copper	0.0125 to 1.25 mg. 0.25 c.c.	27 tumors; mortality 29% (8 died)	4 grew well, 1a, 1c, 2d; 15%. 8 were retarded, 2a, 3c, 3d; 30%. 15 retrogressed, 3a, 6c, 2d, 4f; 55%.
Controls	.....	30 tumors	1 did not grow, 1c; 3%. 29 grew well, 7a, 12c, 7d, 3f; 97%.
Hirudin	0.125 to 1.25 mg.	60 tumors; mortality 15% (9 died)	18 grew well; 30%. 32 were retarded; 53%. 2 retrogressed; 3%. 10 doubtful; 17%.

In the results we state the number of tumors in each class (a, c, d, f) of tumors.

Mice were injected with a mixture of 0.25 of a cubic centimeter of colloidal copper and 0.0125 of a milligram to 1.25 milligrams of hirudin, the smaller dose of hirudin being injected first, and the dose being gradually increased in the course of the following injections; while in the mice injected with hirudin alone 30 per cent. of the tumors grew normally, 17 per cent. were doubtful, 53 per cent. were retarded, and only 3 per cent. retrogressed (the latter being in this case included among the non-growing tumors); only 15 per cent. of tumors grew normally among the mice treated with the combination of hirudin and colloidal copper; 30 per cent. were retarded, and 55 per cent. retrogressed. Among this latter lot of mice there had been

a mortality of 29 per cent. (only the mice living to the end of the experiment being included in the list); among the mice injected with hirudin only, the mortality was smaller; *viz.*, 15 per cent. We see, therefore, that the combination of hirudin and colloidal copper is much more effective than either substance given alone.

These additional investigations confirm, therefore, our previous experiments, and we may now with still greater certainty draw the general conclusions which we mentioned in our previous paper on immunization against the action of substances inhibiting tumor growth.

#### SUMMARY.

Our later investigations confirm our previous results.

1. It is possible to increase markedly the effect of substances inhibiting tumor growth by using certain combinations of these substances which, when given alone, have some effect on tumor growth.

2. Immunity acquired against the effect of these substances depends partly upon an active immunization of the tumor cells themselves against the action of these substances, and this immunity is transmitted to the following generations of tumor cells.

3. The immunity against the substances inhibiting tumor growth is, as far as we have investigated the problem, specific.

4. Our later experiments provide a more secure basis for the additional and more general conclusions which we mentioned tentatively in our previous paper.

## A COMPARISON OF AUTOPLASTIC AND HOMEOPlastic TRANSPLANTATION OF THYROID TISSUE IN THE GUINEA PIG.\*

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It is our purpose in this work, undertaken at the suggestion and carried out with the aid of Dr. Leo Loeb, to trace the fate of the thyroid gland after homeoplastic transplantation, and to compare it with the behavior of this tissue after autoplasmic transplantation.

The vast literature of thyroid transplantation relates chiefly to autoplasmic transplantation. A few brief statements on homeotransplantation are made by Cristiani (1). An accurate comparative analysis of the difference in the growth of auto- and homeoplastically transplanted thyroid tissue does, however, not exist. A communication, published recently by Leischner and Köhler (2) mentions only the disappearance and resorption of the homeograft, without giving histological descriptions.

The findings of the various authors, as far as they concern the fate of thyroid tissue after autotransplantation, agree in general. Von Eiselsberg's (3) successful implantation of cat thyroid (1892), with an histological description, was in all points confirmed by Sultan (1898) (4). The work of Enderlen (1898) (5) deals with the successful implantation of the thyroid as an established fact and takes up the further question, whether the transplanted gland continues to exert its normal function, and whether it produces and eliminates a sufficient quantity of colloid. Enderlen concludes that while the transplanted gland produces colloid, it does so in insufficient quantity, and that the elimination of the colloid is insufficient.

The same question concerning the secretion of colloid on the part of the transplanted gland was raised again in 1906 by Payer (6), who transplanted thyroids of dogs, cats, rabbits, and guinea pigs into the lower pole of the spleen, which he considers favorable soil; he finds an extensive elimination of colloid, taking the presence of colloid in the lymphatic vessels of the spleen as proof of this elimination.

Salzer (7), who transplanted thyroid into the abdominal musculature, considers this tissue likewise favorable soil for the absorption of colloid. He states that in some places the muscle tissue directly surrounds the thyroid vesicles; that in other places, however, the implanted gland is surrounded by a very fine connective tissue capsule; at no place was Salzer able to find a more or less dense connective tissue layer surrounding the implanted graft.

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Numerous papers deal with technical questions in addition to the histological description; for instance, the soil most favorable for transplantation. Thus Cristiani writes exclusively on questions of technique (over thirty papers, 1894-1906); he discusses especially the size of the transplanted piece and the importance of the capsule for successful transplantation.

In his first work Cristiani used the same technique for subcutaneous or intraperitoneal transplantation as his predecessors; in his later work he gives preference to subcutaneous transplantation into the ear of the rabbit, as this method permits better observation of the size, transparency, and vascularization of the graft. Salzer prefers intramuscular transplantation into the abdominal wall; he considers this method preferable to the one recommended by Serman and Kocher (1908) (8) of transplantation into the metaphysis of the femur.

Von Eiselsberg (3) already gives as important factors for successful transplantation asepsis and rapid transfer (within ten seconds) of the still warm, living tissue. Cristiani found that exposure of the pieces to the air for ten seconds does not injure the tissue, but that a longer exposure markedly diminishes its vitality. Cristiani successfully carried out autotransplantations in mammals, birds, reptiles, and amphibia; in fishes he did not succeed on account of technical difficulties. According to Cristiani, the thyroid continues to live and to grow after autoplasmic transplantation; even the transplanted thyroid, if transplanted a second time, grows again, provided the transplantation had not been undertaken at too early a date.

Cristiani found, furthermore, a remarkable power of resistance of the transplanted thyroid against different infections with various microorganisms (*B. coli*, streptococcus, and staphylococcus); even different substances, such as turpentine, usually causing necrosis, did not injure the transplanted graft.

In this laboratory successful transplantations of the thyroid were obtained by Carroll Smith (9) in connection with a different problem. He describes, however, only the autoplastically transplanted pieces, without reference to the behavior of the corresponding homeografts.

#### METHOD.

In our experiments we used guinea pigs, working always on two animals. The neck and abdominal region of the animals are shaved and cleaned with alcohol and a solution of mercury bichloride, then covered with sterile towels soaked in the mercury bichloride solution, and the animals are narcotized with ether. An incision 1 to 2 cm. in length is made in the abdominal region. Into this incision an instrument is introduced and narrow pockets are made on the right and left sides between the skin and subcutaneous tissue or the abdominal musculature, at a distance of about 3 to 4 cm. from the point of incision.

Our series includes over 75 animals with more than 140 grafts. Pieces of the remaining part of the thyroid gland were examined microscopically. The animals were killed with chloroform at intervals of 24, 36, 48, and 72 hours, and 4, 5, 7, 9, 11, 12, 13, 14, 16, 17, 19, 22, 26, 27, 29, 31, 33, 40, 43, 47, and 52 days after the trans-

plantation; the grafts and the parts of the remaining thyroid lobe were preserved in Zenker's fluid and cut serially.

#### DESCRIPTION OF THE TRANSPLANTS.

Transplanted tissue removed 24, 36, and 48 hours after transplantation was necrotic, with the exception of a narrow peripheral zone. Towards the periphery numerous widely dilated capillaries with an occasional fibroblast between them are seen, penetrating into the central necrotic area. The peripheral zone consists largely of more or less well preserved follicles with epithelium and colloid, which appears retracted and lying free in the lumen of the follicle. The epithelium is flat or cuboidal; its nuclei are vesicular and rather dark. Mitoses are absent. In the surrounding tissue isolated fibroblasts, lymphocytes, and polynuclear leucocytes are seen. The grafts lie free in the pockets. There is at this period no difference between the auto- and homeograft.

*After 72 Hours.*—An extensive central necrotic area is still present, but it is now divided into smaller parts by a wide network of capillaries. The number of chromatin particles and pycnotic nuclei in the necrotic material is diminished, while the fibroblasts, polynuclear leucocytes, and lymphocytes are increased. The peripheral living follicular zone has become larger and consists of two to three rows of follicles, lined with high cuboidal epithelium, containing light vesicular nuclei, which already show some, although not yet numerous mitotic figures. Colloid is present in the majority of the follicles. At several places fibroblasts from the periphery are invading the spaces between the follicles; they originate from the host tissue and are very numerous in the tissue surrounding the grafts; mitoses are quite frequent in these cells. There is not yet any distinct difference between auto- and homeotransplant, although the blood vessel supply in one homeograft is smaller.

*After 4 Days.*—A central necrotic area is still present, but the detritus has much decreased in quantity; the chromatin particles and pycnotic nuclei are less numerous. Dilated capillaries, accompanied by fibroblasts, containing mitoses, are numerous in the necrotic area. Lymphocytes and polynuclear leucocytes are met only here and there. Towards the periphery we meet larger follicles with flat epithelium, with small, moderately light nuclei and solid colloid. Some small new follicles are found in the periphery, which are either entirely isolated or united in groups of two or three. Their epithelium is high cuboidal; the nuclei are large and vesicular, some containing mitoses. Colloid is absent in the smaller follicles. A few isolated, solid cell nests, consisting of six to ten circularly arranged cells, are also seen; several show already the beginning of a central lumen. Numerous fibroblasts, frequently with mitoses, are penetrating from the surrounding tissue in several directions into the transplanted tissue. Lymphocytes and polynuclear leucocytes are infrequent. Auto- and homeotransplant are identical at this period.

*After 5 Days.*—Both grafts are partly and only very loosely adherent to the surrounding host tissue; such adhesions become more and more marked in the following days and are very strong after the 12th day; after this stage the removal of the host tissue together with the graft becomes therefore necessary.

After 5 days what was formerly the central necrotic part is still persisting in the autograft, but it is now much smaller than in the earlier stages and is represented chiefly by dilated capillaries surrounded by numerous fibroblasts which have invaded this area. The number of colloid-bearing follicles has increased very much and this follicular zone occupies now a relatively much larger part of the section. Small solid cell areas and short tortuous tubules with high cylindrical epithelium and with very closely arranged nuclei are seen more frequently at this period; they are situated either in the periphery or further towards the center; these cell tubules show frequently evagination into the surrounding tissue as well as papillary protrusions into the lumen. Various stages of the separation of small round follicles from these tubules can be seen. Mitotic figures have become numerous; frequently as many as eight can be seen in one field. Colloid is absent in the small follicles. Lymphocytes and polynuclear leucocytes are seldom seen; on the other hand, fibroblasts are frequent, especially in the peripheral parts and in the surrounding tissue, from where they invade the grafts arranged in bundles and in different directions.

In a corresponding homeograft the same conditions prevail in the periphery, but the larger part of the section is occupied by an extensive area of extravasated blood. Another homeograft 5 days old, however, differs already from the corresponding autograft. (1) In the former a much larger number of lymphocytes is present, which protrude into and between the follicles and begin to cause the destruction of the follicles. In some places they form small accumulations; they are seen in the larger blood vessels and in the connective tissue from where they penetrate towards the follicles. (2) The number of fibroblasts is greater in the homeograft, and their arrangement is at several places less loose than in the autograft; in dense bundles they invade the tissue between the follicles, compressing several of them. But this destruction is as yet only very slight; there is merely an indication of it present. In one homeograft are seen even especially long tortuous cell cords, consisting of two rows of closely adjoining cylindrical cells which in some places separate to form a narrow lumen. In oblique sections they appear as isolated small cell nests or groups of cell nests, with numerous mitoses. In cross-sections they represent oval or round follicles with cylindrical epithelium.

*After 7 Days.*—The central part of the autograft, consisting in some places chiefly of blood vessels, is still distinct, but much smaller than at the earlier stages. The number of fibroblasts in this part is also markedly increased, but their arrangement is very loose. Lymphocytes are rare and found chiefly in the blood vessels. The old transplanted blood vessels are nearly entirely occluded through the proliferation of cells derived from the blood vessel walls. The central connective tissue contains numerous brownish yellow blood pigment cells. The number of follicles with high cuboidal epithelium is large; they are mostly round or oval and not compressed. We find also tubular epithelial structures with irregular evaginations and some mitoses. Several follicles contain blood and desquamated cells. The graft is surrounded by a rather dense connective tissue.

There is after 7 days a distinct difference between the homeo- and autograft. In the former the number of follicles and the amount of colloid is markedly decreased; the preserved follicles, which are present only at the periphery, are compressed by connective tissue, which invades the spaces between them; the follicles are frequently smaller and flatter than in the autograft and contain more rarely colloid, but more frequently desquamated cells. Usually the epithelium is of normal height and may also contain mitoses. As in the grafts 5 days old, there are numerous lymphocytes in the connective tissue and around the follicles, into which they migrate, causing their destruction. In addition, we find between the individual follicles a larger number of densely arranged connective tissue cells with mitoses in the process of producing fibrous tissue. The greater part of the graft, especially its central parts, consists of a densely built connective tissue, which in some places appears hyaline; it contains also lymphocytes and traces of destroyed or compressed follicles, which appear as narrow clefts, containing several desquamated cells. The graft is surrounded by a dense connective tissue with numerous mitoses in the fibroblasts.

*After 9 Days.*—The difference between auto- and homeograft is distinct. In the autograft a larger number of well preserved colloid-containing follicles, the epithelial cells of which contain numerous mitoses, is present; these follicles are separated by a thin layer of connective tissue. Lymphocytes do not invade the epithelium. The central parts of the pieces consist of loosely arranged connective tissue cells with light vesicular nuclei; there are also present numerous brown blood pigment cells. Isolated small or large solid cell nests, as well as small groups of small empty follicles, are frequent in the central areas. In some places the cell nests are strongly developed, and in such cases a large number of mitoses may be seen in the epithelial cells; as, for instance, eight to fifteen in one field. Lymphocytes are rarely present and usually in small isolated nests. Polynuclear leucocytes are occasionally seen in the larger blood vessels. The graft is surrounded by a moderately dense connective tissue.

In the homeograft only isolated follicles, separated and strongly compressed by surrounding connective tissue bands, are seen. They are found mostly in the periphery. The larger part of the follicles in the homeograft is destroyed by lymphocytes; this destruction can be followed especially distinctly in the case of the isolated central follicles; these are surrounded by numerous lymphocytes, which invade the follicles, and penetrate between the epithelial cells, destroying them. The preserved follicles have a flat epithelium with compressed nuclei, which rarely contain mitoses. A few follicles contain solid colloid. Large areas of the sections are occupied by lymphocytes, which fill also the larger blood vessels. The transplanted parathyroid shows the same destruction through lymphocytes as the thyroid tissue. The central parts of the graft consist partly of dense masses of fibroblasts, with distinct nuclei, which in some places form a fibrous, nearly hyaline tissue. In the center also lymphocytes are numerous, forming rather large accumulations and filling the larger blood vessels. The original transplanted blood vessels show a totally obliterated lumen and a remarkably thickened wall, in the cells of which mitoses may be visible. Rarely an isolated follicle with cuboidal epithelium, occasionally infiltrated by lymphocytes, is seen in the central parts. Some follicles do not contain any colloid, but only desquamated cells. Blood vessels are rarely seen. The graft is surrounded

by densely built connective tissue, which penetrates widely into the interior of the section.

*After 11 Days.*—In the autograft the number of follicles with cuboidal epithelium is increasing; they are now seen not only in the peripheral, but also in the central zones, either isolated or combined into small groups. Formation of papillæ and invagination and evagination of the epithelium are frequent in these follicles; furthermore, small new follicles with numerous mitoses and without colloid seem to develop from the larger tubes. Numerous follicles are filled with red corpuscles, with lymphocytes and polynuclear leucocytes among them. Between the follicles are fibroblasts, but their number is small and they are loosely arranged; they contain occasionally a mitotic figure. Lymphocytes are absent; polynuclear leucocytes are found only in a very limited number; but brownish blood pigment cells are frequent. The blood vessel supply of the graft is extensive. The central former necrotic area is now much smaller and consists of a net of capillaries, filled with red blood cells; between the capillaries are numerous isolated fibroblasts; several lymphocytes can also be seen, but polynuclear leucocytes are more frequent. Near the thyroid, and separated from it only by a narrow layer of loosely arranged fibroblasts, parathyroid tissue is seen, which appears entirely normal, with the exception of the presence of a few fibroblasts. The entire graft is surrounded by a ring of densely arranged fibroblasts, which in several places are compressing each other and appear narrow; in other places the arrangement is looser, the nuclei are round and vesicular, with two to three mitoses in a field. Lymphocytes and polynuclear leucocytes are rare.

After 11 days the difference between auto- and homeograft is very distinct; in the latter the number of preserved follicles is diminished; the follicles that are left are smaller; the epithelium is often flat, with elongated nuclei, which rarely show mitoses. Colloid is present in the majority of follicles, but it is retracted, solid, and without vacuoles. Lymphocytes and polynuclear leucocytes are not seen frequently. In several places fibroblasts are invading in loose bundles the graft between the follicles. Brown blood pigment cells are frequent. The larger central part of the graft consists of a granular mass stained reddish with eosin—the remnants of the necrotic parts of the transplanted tissue and of fibroblasts which have penetrated into it. Lymphocytes and leucocytes are absent. The blood supply is limited. Numerous phagocytic connective tissue cells are present in the necrotic material.

*After 12 Days.*—Both grafts are moderately firmly adherent to the adjacent tissue. Microscopically the autograft appears similar to the one excised 11 days after transplantation. There are seen numerous round and oval follicles, located mostly at the periphery. While colloid is filling the majority of the follicles, several are empty and others contain desquamated cells with pycnotic nuclei. The larger part of the graft is occupied by a loosely built connective tissue, in which is occasionally embedded a follicle with high cuboidal epithelium. Several foreign body giant-cells are present. Polynuclear leucocytes are here only rarely seen, but they are more frequent than lymphocytes. The graft is now, and usually also at later periods, surrounded by a densely built connective tissue. The homeograft after 12 days is very different. Only a small number of follicles with insignificant remnants of colloid is present. They are usually found in the periphery and their epithelium is partly preserved, partly desquamated, and

with a protoplasm which is often vacuolar; the nuclei are vesicular and frequently indented. Most of the follicles are markedly compressed by connective tissue fibers and show only a narrow lumen. Frequently lymphocytes surround the follicles, invading and destroying the epithelium. The preserved follicles are separated from each other by dense hyaline connective tissue, in which remnants of cell detritus can be seen; a small number of lymphocytes and polynuclear leucocytes are also present. The graft is, as in the future periods, surrounded by a dense connective tissue, containing many lymphocytes.

*After 13 Days.*—The number of follicles filled with colloid is increased in the autograft; they are lined with cuboidal epithelium, containing vesicular nuclei, with occasional mitoses. In general the number of mitoses is now smaller than in an earlier period; namely, up to the 9th day. Occasionally small solid cell areas are seen. The numerous follicles are located here as in the normal gland close to each other, divided by scanty connective tissue. Lymphocytes and polynuclear leucocytes are absent. The central part of the graft, which is without follicles, is relatively very small and consists of loosely arranged fibroblasts and brown blood pigment cells. Blood vessels are frequent.

The difference between auto- and homeograft is sharp after 13 days. In the homeograft only a few peripheral follicles are present; the height of the epithelial cells varies, with a tendency to become lower. Mitoses are rare, and frequently the epithelial cells are desquamated. Colloid is present only in small amounts; but the lymphocytes, which invade and destroy the follicular epithelium, are frequent. A few polynuclear leucocytes are also present. The follicles are separated from each other by rather broad connective tissue septa, which appear frequently hyaline. These septa completely compress and secondarily destroy the follicles. Lymphocytes are present in large numbers and frequently form large nests. The center is filled with a dense, sometimes hyaline connective tissue, in which elongated nuclei and a small number of lymphocytes are present; the latter also fill the blood vessels. The old transplanted blood vessels are totally obliterated and their wall is thickened.

*After 14 Days.*—The conditions are analogous to those existing after 13 days.

*After 16 Days.*—Nearly the entire section of the autograft consists of closely arranged small and large follicles with cuboidal epithelium and light vesicular nuclei, which show only rarely a mitotic figure. Vacuolar colloid fills nearly all the follicles; occasionally one sees embedded in the colloid a desquamated cell or an extremely large, swollen vesicular nucleus, with chromatin near the membrane. Lymphocytes and polynuclear leucocytes are absent. Only in a very small part of the center of the section follicles are absent; where they are absent we find numerous connective tissue cells, which may form a loose fibrillar tissue, containing blood vessels, several foreign body giant-cells, isolated lymphocytes, and numerous brown blood pigment cells.

The two corresponding homeografts differ strongly from the autograft, and also slightly from each other. In one case about four to six partly preserved follicles are present, containing a number of desquamated epithelial cells; numerous lymphocytes surround the follicles, invade the epithelium, and destroy it. Small remnants of colloid are present. The few preserved follicles are separated from each other by broad hyaline connective tissue septa, compressing the follicles, which assume an elongated shape. The larger part of the section con-

sists of a central dense connective tissue with numerous lymphocytes. Far better preserved appears the second homeograft of the same age; here a larger number of follicles with preserved epithelium and colloid is present; the epithelium and nuclei, which still show mitoses, are of the same character as in the autograft, but in the homeograft connective tissue is invading the spaces between the individual follicles, separating them from each other. The invading connective tissue contains a large number of lymphocytes. The arrangement of the follicles is not regular, they are found not only along the periphery, but isolated ones are also seen in the central parts; they are smaller and do not contain any colloid, but even here a mitotic figure is seen in the epithelium. These isolated follicles are compressed by broad connective tissue bands filled with lymphocytes. A number of follicles are destroyed by the invading lymphocytes, but this process of destruction in the second piece is not as marked as in the majority of the homeografts. The homeograft is divided by broad septa into several lobules; a central large hyaline area is lacking. In the central connective tissue mitoses are not infrequent; lymphocytes are numerous, but they are not found accumulated in small clusters. The graft is surrounded by dense connective tissue with lymphocytes, which are also seen in the adjacent tissue together with giant-cells.

*After 17 Days.*—The autografts show the typical picture of a normal thyroid with closely arranged follicles, lined with epithelium, which surround colloid. The epithelium is high cuboidal, with large, vesicular nuclei, containing rarely a mitosis. Lymphocytes are absent. In a few follicles a slight desquamation, extravasated blood, and blood pigment are found. The small center consists of loosely arranged connective tissue cells, which do not show anywhere the tendency to form a fibrous or hyaline tissue; the larger blood vessels are also located here. The graft is surrounded only partly by dense connective tissue, partly by muscle fibers.

In the corresponding homeograft we observe the results of tissue destruction; almost no follicles are found anywhere, only remnants of destroyed follicles can be seen; they appear as narrow fissures filled with several desquamated cells and lymphocytes. The rest of the graft shows a rather uniform hyaline area, with distinct nuclei; lymphocytes are also present in limited number, located mostly in the periphery, at the place of the former follicles. Several giant-cells are met along the periphery. The larger central blood vessels are totally obliterated and their wall is thickened. The blood supply of the graft is limited; the blood vessels are located mostly in the periphery. The grafts are surrounded partly by a fibrous connective tissue, partly by muscle tissue, containing giant-cells.

*After 19 Days.*—The regeneration in the autograft is nearly complete. Follicles of various sizes are lying close to each other; the epithelium is somewhat flatter than after 15 and 17 days and the nuclei do not show any mitoses. All follicles are entirely filled with colloid. Lymphocytes are absent. Connective tissue is scant, so that a connective tissue center can hardly be made out. The connective tissue which does exist, however, consists of loosely arranged fibrils with light vesicular nuclei, numerous brownish blood pigment cells, blood vessels, and lymphocytes. Both homeografts show conditions similar to those after 16 days. While in one piece only fibrous connective tissue with several giant-cells

is seen, numerous colloid-filled follicles are still preserved in the other homeograft. The follicles are lined with a cuboidal epithelium, containing as before light vesicular nuclei; the follicles are mostly isolated, surrounded by connective tissue, which in some parts is finely fibrillar, but forms in other places dense fibrous bands, which divide the section into several small parts. This fibrous connective tissue encircles frequently isolated follicles or groups of follicles, compressing them, so that their lumen is represented only by a narrow cleft. The connective tissue is accompanied by numerous lymphocytes, which invade and destroy the epithelium of the follicles. In many places, especially towards the center, the connective tissue septa are broader and the number of follicles between them diminishes steadily; near the center the septa, containing lymphocytes coming from all directions, unite; between them are seen partly destroyed follicles with desquamated cells and elongated spaces, filled with lymphocytes, which probably represent former follicles which have been destroyed. Blood vessels are not numerous, occupying mostly the extreme margin of the graft, which is surrounded by fibrous connective tissue with lymphocytes.

*After 22 Days.*—The typical picture of the normal thyroid gland is still more pronounced in the autograft; the follicles are larger, especially at the periphery, the epithelium is cuboidal, and the nuclei are light and vesicular, without mitoses. The homeografts, analogous to those after 16 and 19 days, show again a small number of preserved follicles with solid particles of colloid; the preserved follicles are smaller than in the autografts, their epithelium and nuclei are normal, but they are actively attacked by lymphocytes, which are found between the epithelial cells of the follicles, invading and destroying the latter. In general the number of lymphocytes is now larger, while after 16 and 19 days the connective tissue prevailed. But here also the connective tissue encircles the follicles, forming broad septa, which divide the section into several parts; this causes the formation of several small hyaline areas, instead of one large central hyaline field. But altogether the number of follicles injured by lymphocytes is more frequent than the ones compressed by connective tissue. Larger and smaller accumulations of lymphocytes are seen in all parts of the sections. Blood vessels, chiefly situated in the periphery, are, on the whole, rare.

*After 26 Days.*—Both autografts show again the typical picture of the normal thyroid, consisting of closely arranged, colloid-filled follicles. In the same graft a perfectly normal parathyroid is present, consisting of solid strands of epithelial cells.

After 26 days remnants of the homeografts could not be made out with certainty; the pieces found consist of a dense connective tissue, containing several giant-cells and a few lymphocytes.

*After 27 Days.*—The picture of the autograft is similar to the autografts 26 days old; perfectly regenerated parathyroid tissue is again present. The homeografts after 27 days are, however, much better preserved than those of the preceding stage. It is surrounded by a dense, moderately broad connective tissue capsule, which sends several septa into the interior of the tissue. The structure of the graft is very dense, as can be seen already under the low power. Along the periphery are found about fifteen to twenty very small round or oval follicles, which are isolated from each other and which still contain solid colloid without vacuoles. In the center only one well preserved follicle is seen. The



epithelium of the preserved follicles is cuboidal with vesicular nuclei. Between the epithelial cells of the follicles lymphocytes are frequently present; they penetrate towards the lumen of the follicles, at the same time injuring the latter. Connective tissue is present in a comparatively small amount; it does not form wide septa which compress the follicles. Lymphocytes, however, are numerous and they accomplish in the main the destruction of the follicles. The blood vessel supply is extremely small in comparison with that of the autograft, where a well developed system of blood vessels is always embedded in the central, loosely built connective tissue, which is entirely absent in the corresponding stages of the homeografts. •

*After 29 Days.*—Both autografts again show the typical structure of the normal thyroid gland. The central connective tissue is very slight, of loose texture, and contains brown blood pigment cells and numerous vessels; lymphatic spaces filled with colloid are also present. The lobular structure of the gland is very distinct; fine septa containing blood vessels traverse the tissue in several directions, without altering the shape of the follicles, or without compressing them. The follicles are very numerous, and of larger size than in the previous stages; their shape is frequently oval or irregular; they are distended with colloid, in which are rarely seen one to two desquamated cells. Well developed follicles filled with colloid are found at several places in the fibrous capsule which surrounds the graft; their shape or size is not changed by the surrounding connective tissue; some show the formation of papillæ. Both homeografts are preserved after 29 days; they show some well preserved follicles, containing colloid. But in both cases a very active destruction of the majority of follicles by lymphocytes and connective tissue is visible, the connective tissue being unequally active in both cases. In the first case wide fibrous septa are passing through the piece, dividing it into isolated lobules, which they surround like a ring; the fibrous tissue invades also the interior of the lobules, surrounding and compressing the individual follicles to such an extent that their total obliteration results. The connective tissue is accompanied everywhere by lymphocytes, which invade and destroy the follicular epithelium; they are found also in large numbers among the desquamated cells. In the second piece a large number of smaller follicles containing colloid is present. Broad connective tissue bands are lacking, but we find long rows of lymphocytes, which penetrate between and into the follicles and destroy them. The blood vessel supply of the homeografts is very scant.

*After 31 Days.*—The autografts show the same picture as after 29 days. One homeograft shows still a remarkable number of well preserved follicles, filled with colloid, while many of them are attacked by lymphocytes; on the whole, the number of lymphocytes is in this case relatively small and the destructive process is mainly accomplished through compression of the follicles by broad septa of hyaline connective tissue. The follicles are mostly narrow, elongated, and arranged in isolated groups, which are surrounded by a hyaline connective tissue and which become totally obliterated as a result of compression. Some parts of the section appear entirely hyaline; in other parts traces of almost completely destroyed follicles can be recognized; they appear as narrow clefts, filled with desquamated cells and lymphocytes. The homeografts are surrounded by a broad capsule of connective hyaline tissue.

*After 33 Days.*—We find in the autografts the same conditions as in the previous stages; there is noticeable a very slight desquamation of cells in several follicles. There are no definite traces of the homeografts at this stage.

*After 40 Days.*—The autografts show markedly larger follicles, especially along the periphery; the epithelium appears flatter, the nuclei smaller; in the larger follicles papillæ are protruding into the lumen. In some follicles a slight desquamation of epithelial cells is observed. A central area of connective tissue can no longer be seen, only near one pole of the piece there is a moderately dense connective tissue with numerous blood vessels, blood pigment cells, several lymphocytes, and a little fat tissue. The graft is surrounded partly by fibrous connective tissue, partly by muscle fibers. In the homeograft we see only fibrous tissue, containing spaces filled with lymphocytes. There are several giant-cells.

*After 43 Days.*—The autografts from three experiments show again the typical picture of the normal thyroid gland. There is nowhere a compression exerted by the connective tissue. The grafts are surrounded partly by moderately dense connective tissue, containing some lymphocytes; in other places they join directly surrounding muscle tissue; a few follicles may even protrude between the muscle fibers. Some fat tissue and numerous blood vessels are also found at the periphery. No trace of the homeografts could be found after 43 days.

*After 47 and 52 Days.*—The same conditions are noted; the autografts are of larger dimensions; the tendency of the follicles to protrude into the neighboring muscle tissue is increasing.

After 47 and 52 days no traces of the homeografts were found. We see, therefore, that in this series the homeografts could not be found after the 33d day.

#### SUMMARY AND CONCLUSIONS.

If we now consider briefly the principal results of our investigation, we can, in comparing the behavior of the thyroid after auto- and homeoplastic transplantation, in general recognize three stages. In the first stage, comprising the first 4 to 5 days after transplantation, there is no noticeable difference between the auto- and homeograft; both corresponding pieces behave in the main alike; large parts of both grafts become necrotic in the center; the necrosis begins shortly after the transplantation and concerns only a part of the periphery; here a narrow zone of thyroid tissue is left; it consists of one to two rows of partly well recognizable follicles. After 48 hours the first fibroblasts, polynuclear leucocytes, and lymphocytes appear in the tissue surrounding the grafts. After 72 hours these elements are present also in the center of the thyroid tissue. At the same date the first mitoses appear in the follicular epithelium; their number is, however, as yet small, but it in-

creases markedly during the following days. After 4 days are found the first newly formed follicles, and from now on their number is increasing. Colloid is present only in small amounts in the old follicles. The central necrotic area still persists, but the masses of detritus are becoming smaller, while the number of fibroblasts and lymphocytes in the necrotic part is increasing; new blood vessels are also visible in the necrotic center.

The second stage, the stage of transition, extends over the next seven days. The earliest, at first slight, differences between the auto- and homeograft appear after 5 days; the difference is usually very definite after 12 days. During this time there is an increase in the number of follicles in the autograft and correspondingly a decrease in the size of the necrotic central area, which is caused by a gradually progressing removal of the necrotic material in the center of the piece. The follicles are lined with a high cylindrical epithelium, which contains numerous mitoses. The number of mitoses increases markedly after the 4th day and reaches a maximum between the 7th and 9th days. Newly produced colloid is always present in the autograft after the 8th day; in the newly formed follicles the colloid contains numerous vacuoles and is almost never retracted, in contradistinction to the solid retracted colloid in the old follicles, which probably was present in the follicles at the time of transplantation. At the end of this period the central part is almost entirely freed from the necrotic masses and is filled with loosely arranged connective tissue cells, between which isolated lymphocytes and polynuclear leucocytes can be found. It contains also numerous blood vessels and brown blood pigment cells.

After 5 days, with the beginning of the second stage, the first as yet rather insignificant differences become noticeable in the homeo-transplants. They are as follows: (1) The number of lymphocytes is much larger in the homeo- than in the autograft. (2) The fibroblasts are increased in the central area and these fibroblasts form here very soon firm connective tissue bundles; they surround also the individual follicles or small groups of follicles and form fibrous bands, which are at this stage still very small. During the following days the number of lymphocytes increases and the fibrous connective tissue becomes more prominent, so that 7, 9, 10, and 11 days after

transplantation numerous follicles are not only destroyed by the surrounding and invading lymphocytes, but in addition a part of the follicles is encircled and compressed by wide connective tissue bands. This compression is especially distinct in the central parts of the homeografts. At this stage the absence of a well developed vascular system is already noticeable in the central parts of the homeografts. It is especially noteworthy that the follicles of the homeograft which escaped destruction by the lymphocytes and connective tissue are as well preserved as in the autograft. Colloid is usually present in smaller amounts than in the autografts, even in the well preserved follicles of the homeografts.

It must, however, be stated that some variations occur in this stage in the degree of destruction of the homeotransplanted pieces. Side by side with pieces in which a marked destruction has taken place, there are other pieces which suffered as yet relatively little. But some of the above mentioned differences between the auto- and homeotransplants are always present to a smaller or larger extent. There are also certain variations in the relative strength, with which lymphocytes and connective tissue injure the follicles; in some homeotransplants the attacks on the part of the lymphocytes prevail, while in others those on the part of the connective tissue are more prominent. The homeotransplants, which are relatively little injured at this stage are spared only to be subject to a stronger attack on the part of the lymphocytes and connective tissue during the following stage.

The third stage begins approximately with the 12th day. From now on the difference between auto- and homeograft is sharply defined. In the autografts the regeneration of the thyroid tissue is steadily progressing and is nearly complete after 21 days. After this date the autograft represents throughout the picture of the normal thyroid gland; well developed follicles filled with colloid are surrounded by a very scant connective tissue. Mitoses, which begin already to decrease after the 9th day, are still present at the end of 17 days, but absent after this date. The small amount of loosely built, usually centrally located connective tissue, has no tendency to undergo a fibrous or hyaline change and does nowhere exert a compression on the follicles. It contains always a large number of blood vessels and only a few lymphocytes.

In the homeograft the secondary destruction of the follicles is progressing with great intensity after the 12th day. In contradistinction to a primary degeneration of the follicles, due to the action of substances circulating in the body fluids, which might have been expected to take place, but which in reality does not occur, and which would be followed only secondarily by a connective tissue proliferation and lymphocytic invasion, we may speak of the destruction by means of lymphocytes and connective tissue, which actually does take place, as a secondary destruction of the follicles. This secondary mode of destruction of the follicles through lymphocytes and connective tissue is found exclusively in the homeotransplant. Following this destruction the larger part of the homeografts is occupied by a fibrous, hyaline connective tissue, in which remnants of destroyed follicles in the form of clefts filled with lymphocytes are seen. In several cases the destruction of follicles through lymphocytes, which accumulate in large numbers around the follicles and destroy them very actively, prevails; in other cases the compression of the follicles through fibrous, hyaline connective tissue predominates; in other cases both factors may be about equally active. The few peripheral follicles which escaped destruction are small, probably as a result of compression through the connective tissue; but they may still show normal epithelium and nuclei and may occasionally contain traces of colloid. Newly formed colloid is not present in the homeotransplants at the third stage. It should also be mentioned that the blood supply of the homeografts, especially after the 12th day, is very much restricted and that the blood vessels are mainly located in the connective tissue surrounding the graft, and not in the central connective tissue, which exists here in a much larger quantity and is much denser than in the autograft. At this stage also we find considerable variations in the extent of the injuries, in the individual follicles. But no piece escapes the attack entirely, and those which remain relatively well preserved for a certain period will be attacked at a later date by the connective tissue, as well as by the lymphocytes of the host.

To summarize briefly the principal result: For a short period of time after operation no difference is seen in the behavior of the thyroid after auto- and homeotransplantation. Very soon, however, a

destruction of follicles begins to take place in the homeografts. This destruction is not caused by a direct primary disintegration or solution of follicles, but depends on the destructive activity of (1) the lymphocytes, and (2) of the connective tissue of the host tissue. The former invade the follicles and destroy them directly; the latter grows into the homeografts in larger quantity than into the autografts. In the former it soon becomes fibrous and hyaline; in the latter it remains cellular. The fibrous connective tissue surrounds and compresses and thus destroys the follicles. In some homeografts destruction by means of lymphocytes, in others by connective tissue, preponderates. The rapidity with which the destruction takes place in different homeotransplants also varies. A much better blood vessel supply develops in the autograft than in the homeograft.

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# NOTES ON THE SUBCUTANEOUS ABSORPTION AND THE QUANTITATIVE ESTIMATION OF CHOLESTEROL.\*

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Cholesterol, now known to be a constituent of practically every tissue of the body, was discovered in gall stones in 1775 by Conradi, and was given its name (cholesterin) by Chevreul in 1815.<sup>1</sup> The pathologist's interest in this substance is aroused by the fact that when isolated chemically it proves to be practically inert, yet it is a constituent of tissues which are continually being broken down and replaced by new tissues, and has manifold functions. What becomes of the cholesterol present in these tissues? Part of it, at least, that is the part which remains after pathological destruction, is found as cholesterol crystals in the necrotic tissues. Thus, in atheroma of the aorta cholesterol crystals are commonly found in abundance, and old hemorrhages or necrotic areas usually contain such crystals. In respect to the absorption of deposits of cholesterol, little is known.

Meyer<sup>2</sup> has described a case of adenomatous cyst of the ovary which ruptured *intra vitam*, causing miliary nodules over the peritoneum, which revealed microscopically giant-cells and cholesterol. Le Count<sup>3</sup> found cholesterol giant-cells in a case of carcinoma of the scalp and a tumor from the scrotum.

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<sup>1</sup> Good general accounts, historical and biological, of the relations of cholesterol are given by Moore, F. C., *Cholesterin: Some Account of its Chemical, Physical and Biological Relations*, *Med. Chron.*, 1907-8, xlvii, 204; and Glikin, W., *Ueber Cholesterine und verwandte Stoffe*, *Biochem. Centralbl.*, 1908, vii, 289, 357. McNee, J. W., *Cholesterin: An Account of Its Relations to Pathology and Physiology*, *Quart. Jour. Med.*, 1913-14, vii, 221.

<sup>2</sup> Meyer, C., *Ueber einen Fall von Fremdkörperperitonitis mit Bildung riesenzellenhaltiger Knötchen durch Einkapselung von Cholesterintafeln. mit Bemerkungen über die verschiedenen Riesenzellenarten*, *Beitr. z. path. Anat. u. z. allg. Path.*, 1893, xiii, 76.

<sup>3</sup> Le Count, E. R., *Cholesterin Giant-Cells*, *Jour. Med. Research*, 1902, vii, 166.

He produced the same histological picture by injecting cholesterol crystals suspended in sterile salt solution into the backs of guinea pigs.

That cholesterol and cholesterol esters of palmitic and oleic acids are re-sorbed on feeding and are found in the blood in increased amounts was shown indirectly by Pribram,<sup>4</sup> who noted that serum containing cholesterol (but not cholesterol esters) has an antihemolytic effect toward saponin solutions, while normal serum does not. Jankau<sup>5</sup> observed that cholesterol subcutaneously injected disappeared microscopically from the site of injection even in a few hours.

In a rather unsatisfactory chemical experiment, Aguilar<sup>6</sup> found that cholesterol was absorbed when injected subcutaneously into a dog. He injected 0.25 gm. of cholesterol dissolved in 2 c.c. of ether, and eight days later killed the dog, shaved the skin at the site of injection, and removed a piece of skin with the underlying connective tissue until no further traces of cholesterol could be made out. The tissue was analyzed for cholesterol by extracting with ether, the ether extract treated with alcoholic potash to saponify the fats, and after evaporation of the alcohol the cholesterol was extracted from the residue with ether, which was then filtered, the ether evaporated, and from the residue the weight of cholesterol was determined. This proved to be 0.127 gm. The animal, therefore, in a period of eight days had absorbed about half the amount injected. He concludes that cholesterol is absorbed in part, or rather diffuses very slowly about the site of injection.

#### SUBCUTANEOUS ABSORPTION.

With a reliable method available for the quantitative determination of cholesterol<sup>7</sup> it was hoped that the absorption of cholesterol from the tissues in crystal form could be more satisfactorily demonstrated and followed chemically at definite intervals step by step as it progressed. With this in mind samples of cholesterol were inserted into the subcutaneous tissues of two series of guinea pigs.

*Series 1.*<sup>8</sup>—Chemically pure cholesterol was placed in a flat tin foil cap (such as is used to cover bottles) and put into a hot air oven at 160° C. until just completely melted, allowed to cool thoroughly, and then, with aseptic precautions, was cut up into small pieces of 0.1 gm. each, accurately weighed.<sup>9</sup> These pieces

<sup>4</sup> Pribram, H., Beitrag zur Kenntniss des Schicksals des Cholesterins und der Cholesterinester im tierischen Organismus, *Biochem. Ztschr.*, 1906, i, 413.

<sup>5</sup> Jankau, L., Über Cholesterin- und Kalkausscheidung mit der Galle, *Arch. f. exper. Path. u. Pharmacol.*, 1892, xxix, 237.

<sup>6</sup> Aguilar, E., Iniezioni di colesterina. Contributo allo studio dell' alimentazione sotto-cutanea, *Boll. d. Soc. di nat. in Napoli* (1908), 1909, series 2, ii, 94.

<sup>7</sup> Corper, H. J., A Modification of Ritter's Method for the Quantitative Estimation of Cholesterol, *Jour. Biol. Chem.*, 1912, xii, 197.

<sup>8</sup> Mr. G. T. Caldwell performed some of the cholesterol analyses in this series.

<sup>9</sup> The pieces of cholesterol were about 1 to 2 mm. thick by 4 mm. wide by 8 mm. long. In some cases it was necessary to use two small pieces of cholesterol to obtain the required 0.1 gm.



were then inserted with aseptic precautions subcutaneously into the backs of guinea pigs, and were removed at certain intervals after insertion for chemical analysis and histological examination.

Histological examination revealed nothing of special interest. The pieces of cholesterol are found well invested in a connective tissue capsule which becomes more mature and firm as the time interval after insertion increases. As we are interested only in the final figures obtained at various periods of removal, the results of the chemical analyses will not be given in detail. It is to be noted that the cholesterol in series 1 was completely melted, so that it formed a solid mass of substance, while in series 2 it was just melted sufficiently to hold together and was, therefore, porous.

- D.<sup>10</sup> Sample removed 15 dys. after insertion yielded 0.095 gm. of cholesterol.  
 A. Sample removed 35 dys. after insertion yielded 0.096 gm. of cholesterol.  
 A. Sample removed 66 dys. after insertion yielded 0.090 gm. of cholesterol.  
 B. Sample removed 120 dys. after insertion yielded 0.090 gm. of cholesterol.  
 B. Sample removed 120 dys. after insertion yielded 0.086 gm. of cholesterol.  
 A. Sample removed 15½ mos. after insertion yielded 0.091 gm. of cholesterol.  
 A. Sample removed 15½ mos. after insertion yielded 0.092 gm. of cholesterol.  
 C. Sample removed 27 mos. after insertion yielded 0.084 gm. of cholesterol.  
 C. Sample removed 27 mos. after insertion yielded 0.079 gm. of cholesterol.  
 C. Sample removed 27 mos. after insertion yielded 0.084 gm. of cholesterol.

It is noted that in this series the removal of the cholesterol from the site of insertion was slight (16 to 20 per cent.) within a period of twenty-seven months, and even this may be questioned, so that we can consider it a rather slow process when the cholesterol is present in appreciable amount and in compact form.

*Series 2.*—Here the samples of cholesterol (0.1 gm., accurately weighed) were much more porous than those implanted in series 1; otherwise the conditions of the experiments were the same.

- H. Sample removed 16 dys. after insertion yielded 0.096 gm. of cholesterol.  
 E. Sample removed 20 dys. after insertion yielded 0.090 gm. of cholesterol.  
 F. Sample removed 9½ mos. after insertion yielded 0.051 gm. of cholesterol.  
 F. Sample removed 9½ mos. after insertion yielded 0.050 gm. of cholesterol.  
 G. Sample removed 11 mos. after insertion yielded 0.049 gm. of cholesterol.  
 G. Sample removed 11 mos. after insertion yielded 0.076 gm. of cholesterol.  
 G. Sample removed 11 mos. after insertion yielded 0.033 gm. of cholesterol.  
 E. Sample removed 13 mos. after insertion yielded 0.068 gm. of cholesterol.  
 E. Sample removed 13 mos. after insertion yielded 0.058 gm. of cholesterol.

As a result of the above experiments it is seen that solid cholesterol is slowly removed from the subcutaneous tissues of the guinea

<sup>10</sup> The capital letters indicate the different guinea pigs; A, B, C, and D (series 1), and E, F, G, and H (series 2).

pig, the rate of absorption being dependent largely upon its physical state of division. It seems probable that the removal of the cholesterol is due rather to absorption than to a mere mechanical action. This removal is probably performed by the fixed tissue cells, of which the giant-cell found around pathological crystals of cholesterol is an expression.

#### QUANTITATIVE ESTIMATION.

In the hope of simplifying the method previously described<sup>11</sup> for the quantitative estimation of cholesterol and thus also reducing the time necessary for performing the analyses, the following experiments were carried out. Instead of the preliminary extraction of the fats of the tissues to be analyzed for cholesterol before saponification, it was thought possible that direct treatment of the tissues with a large excess of sodium alcoholate on the water bath, the excess to be removed by means of carbon dioxide, would prove sufficient, the rest of the method remaining the same.

*Experiment 1.*<sup>12</sup>—Two samples of 30 gm. each of rabbit liver (moist weight) were taken; one was directly saponified with 75 c.c. of 5 per cent. sodium alcoholate, and the other was thoroughly extracted with alcohol and ether, and this extract was saponified with 75 c.c. of 5 per cent. sodium alcoholate; from this point both were carried through the method as previously described. The method of direct saponification of the tissue yielded 0.120 gm. of an oily, yellow product, whereas the alcohol-ether extract yielded 0.083 gm. of pale yellow crystals.

*Experiment 2.*—Duplicate of experiment 1, with 25 gm. of rabbit liver (moist weight). The method of direct saponification of the tissue yielded 0.079 gm. of an oily, yellow product, while the alcohol-ether extract yielded 0.069 gm. of pale yellow crystals.

*Experiment 3.*—Duplicate of experiments 1 and 2, with 30 gm. of rabbit liver (moist weight), yielded 0.162 gm. of an oily, yellow product by the direct method and 0.108 gm. of pale yellow crystals by the alcohol-ether method.

*Experiment 4.*—Two samples of 15 gm. each (moist weight) of rabbit brain were taken; one was directly saponified by means of 65 c.c. of 5 per cent. sodium alcoholate, and the alcohol-ether extract of the other by means of 30 c.c. of 5 per cent. sodium alcoholate. The former yielded 0.438 gm. of an oily, brown product, while the latter yielded 0.682 gm. of pale brown crystals.

*Experiment 5.*—Duplicate of experiment 4, with two samples of 20 gm. each (moist weight) of rabbit brain. The directly saponified tissue (with 75 c.c. of

<sup>11</sup> Corper, H. J., *loc. cit.*

<sup>12</sup> In the following individual experiments the samples of liver, brain, and spleen were not the same, except where stated.

5 per cent. sodium alcoholate) yielded 0.581 gm. of a brownish, slightly crystalline product, and the alcohol-ether extract yielded 0.629 gm. of a crystalline, brown product.

*Experiment 6.*—Duplicate of experiment 4, with two samples of 5 gm. each (dry weight) of cow brain. The directly saponified tissue, with 75 c.c. of 5 per cent. sodium alcoholate, yielded 0.561 gm. of a brown product, and the alcohol-ether extract, with 50 c.c. of 5 per cent. sodium alcoholate, yielded 0.699 gm. of a crystalline, pale brown product.

*Experiment 7.*—Duplicate of experiment 4, with 4 samples of 1 gm. each (dry weight) of cow brain. The two samples directly saponified, with 50 c.c. of 5 per cent. sodium alcoholate, yielded 0.110 and 0.047 gm. of product, while the two alcohol-ether extracted products, with 50 c.c. of 5 per cent. sodium alcoholate, yielded 0.106 and 0.108 gm.

*Experiment 8.*<sup>13</sup>—Two samples of 30 gm. each (moist weight) of steer spleen were taken. One directly saponified, with 75 c.c. of 5 per cent. sodium alcoholate, yielded 0.128 gm. of a crystalline, pale yellow product; and the alcohol-ether extract, saponified by means of 75 c.c. of 5 per cent. sodium alcoholate, yielded 0.150 gm. of pale yellow crystals.

*Experiment 9.*—Duplicate of experiment 8, with two samples of 10 gm. each (dry weight). The tissue directly saponified with 100 c.c. of 5 per cent. sodium alcoholate yielded 0.197 gm. of pale yellow crystals, while the alcohol-ether extract, saponified with 35 c.c. of 5 per cent. sodium alcoholate, yielded 0.264 gm. of pale yellow crystals.

*Experiment 10.*—Duplicate of experiment 9, with two samples of 10 gm. each (dry weight). The tissue directly saponified with 50 c.c. of 5 per cent. sodium alcoholate yielded 0.149 gm. of pale yellow crystals, while the alcohol-ether extract, saponified with 35 c.c. of 5 per cent. sodium alcoholate, yielded 0.233 gm. of pale yellow crystals.

Thinking that possibly better results might be obtained by using a strong watery solution of sodium hydroxide directly on the tissue for saponifying, as is used by Pflüger in his method for determining glycogen, and then proceeding with the rest of the method as originally described (passing through the solution carbon dioxide, etc.), we carried out two analyses.

*Experiment 11.*—Two samples of 10 gm. each (dry weight) of steer spleen were taken, one directly treated with 10 c.c. of water and 50 c.c. of 50 per cent. sodium hydroxide, heated on the water bath for 30 minutes, cooled, carbon dioxide passed to saturation, etc., yielded 0.137 gm. of pale yellow crystals, while the control, carried through the regular alcohol-ether extraction method, yielded 0.250 gm. of pale yellow crystals.

<sup>13</sup> The tissue residues of the spleen in experiments 8, 9, and 10 after alcohol-ether extraction were saponified after addition of absolute alcohol, by means of 100 c.c. of 5 per cent. sodium alcoholate, and then carried through the regular steps of the process for the estimation of cholesterol, but no products were obtained.

*Experiment 12.*—Two samples of 5 gm. each (dry weight) of steer brain, one saponified with 10 c.c. of water and 50 c.c. of 50 per cent. sodium hydroxide, yielded 0.568 gm. of pale brown crystals, while the control yielded 0.720 gm. of pale brown crystals.

As seen from the above experiments, it is impossible to shorten the procedure for the quantitative estimation of cholesterol, as previously described, by directly saponifying the original tissues, rather than the alcohol-ether extract, by means of a large excess of 5 per cent. sodium alcoholate or by means of a strong solution of sodium hydroxide (50 per cent.).

#### GENERAL SUMMARY.

Cholesterol, in 0.1 gram amounts, melted in a compact mass and inserted into the subcutaneous tissues of the back of the guinea pig, is slowly removed, as determined by chemical analyses. The rate of removal is probably greatly dependent upon the physical state of the cholesterol.

The quantitative estimation of cholesterol by the method previously described<sup>14</sup> can not be shortened and simplified by the direct saponification of the tissues by means of 5 per cent. sodium alcoholate or a strong (50 per cent.) solution of sodium hydroxide, instead of saponifying the alcohol-ether extract.

<sup>14</sup> Corper, H. J., *loc. cit.*

# THE RESISTANCE OF PUPS TO LATE CHLOROFORM POISONING IN ITS RELATION TO LIVER GLYCOGEN.\*

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In a former paper<sup>1</sup> it was shown that the various hemorrhagic diseases of the new-born, known as Buhl's disease, Winckel's disease, and melena neonatorum, are probably all expressions of an asphyxial process. This conclusion was based on the following points: (1) the similarity of the anatomical and clinical findings to those known to be induced by lack of oxygen, *viz.*, cyanosis, edema, fat infiltration, hemorrhages, etc.; (2) the experimental production of the various disease pictures by subjecting pregnant animals to the influence of chloroform, a drug which is known to suppress oxidations; (3) the production likewise of similar conditions by direct asphyxiation of the fetuses through ligation of blood vessels to the uterus.

Most of the experiments were made with guinea pigs, but a few dogs were used also.

While the paper was in press, an article by Whipple<sup>2</sup> appeared, in which he showed that in experiments upon four litters of pups he was unable to produce a central necrosis of the liver lobules during the first three weeks of life by the administration of chloroform for a period of approximately two hours. A necrosis of the central portion of the liver lobule is a nearly constant result of a two hour chloroform anesthesia of an adult dog. A portion of the work incorporated in Whipple's article had already been included in one of his former articles.<sup>3</sup> We had, therefore, in our earlier article made note of the absence of findings in Whipple's experiments and had made the suggestion that perhaps a high content of liver glycogen had been concerned in protecting the pups which he used.

\* Received for publication, October 31, 1914.

<sup>1</sup> Graham, E. A., The Pathogenesis of the Hemorrhagic Diseases of the New-Born, *Jour. Exper. Med.*, 1912, xv, 307.

<sup>2</sup> Whipple, G. H., Insusceptibility of Pups to Chloroform Poisoning during the First Three Weeks of Life, *Jour. Exper. Med.*, 1912, xv, 259.

<sup>3</sup> Whipple, G. H., and Hurwitz, S. H., Fibrinogen of the Blood as Influenced by the Liver Necrosis of Chloroform Poisoning, *Jour. Exper. Med.*, 1911, xiii, 136.

However, since in our experiments no central liver necrosis was found, although the livers became very fatty, no claim was made that the changes found justified the diagnosis of a true chloroform poisoning, since the presence of a central necrosis has been regarded by some as characteristic of the picture. We felt that perhaps they might be expressions merely of an interference with the fetal blood supply induced by changes in the placenta, of the kind which had already been described by Whipple. Nevertheless, it has seemed desirable to investigate the point further, because from a theoretical standpoint it would be of interest to determine the nature of the relative insusceptibility of pups to late chloroform poisoning. Accordingly, we have carried out experiments with this point in view.

We have been able to corroborate the main conclusion of Whipple that pups are relatively insusceptible to the production of liver necrosis by chloroform. But we feel that the evidence is practically conclusive that this greater resistance is dependent chiefly, if not entirely, on the high glycogen content of the livers of normal, well nourished pups. Our evidence, briefly summarized, is as follows: 1. Pups were made to show the typical liver necrosis, if given chloroform, after adopting measures to deplete glycogen, either by (a) starvation or (b) phlorhizinization and starvation. 2. In adult animals it is well known that increasing the glycogen content of the liver by feeding of carbohydrates serves to protect against poisons which, like chloroform, produce fatty changes in the liver; *e. g.*, phosphorus, arsenic, etc. 3. An examination of the liver of a pup twenty-four hours old showed that as much as 9.07 per cent. of its total weight was glycogen.

It is interesting to compare this amount with the amounts found by Schöndorff<sup>4</sup> in a series of eight adult dogs in which an attempt was made to raise the glycogen content to a maximum by feeding for several days with a large quantity of carbohydrate. The results represent the percentages of the total weights of the livers, and are as follows: 4.3, 7.6, 18.7, 17.1, 16.4, 9.9, 7.3, and 15. Thus it is seen that our finding in the pup's liver of 9.07 per cent. is greater than or equal to the amounts found in half the adult dogs in which an attempt had been made to crowd the liver with glycogen. In other words, this value of 9.07 per cent. may be considered as indicating a liver that is well filled with glycogen.<sup>5</sup>

<sup>4</sup> Schöndorff, B., Ueber den Maximalwerth des Gesammtglykogengehalts von Hunden, *Arch. f. d. ges. Physiol.*, 1903, xcix, 191.

<sup>5</sup> A number of analyses made by Demant (Ueber den Glycogengehalt der Leber neugeborener Hunde, *Ztschr. f. physiol. Chem.*, 1887, xi, 142) on the livers of pups show even higher amounts than the one obtained by us. Thus the livers of three pups, aged 1 hour, 3½ hours, and 3 hours, respectively, showed, by Brücke's method, percentage values of glycogen of 11.389, 9.527, and 5.443, re-

Roger<sup>6</sup> long ago recognized the detoxicating action of the liver against strychnin. Since then it has been learned that the liver exercises a similar action against many other poisonous substances, and that this protective property of the liver is chiefly dependent on glycogen. Rosenfeld<sup>7</sup> has shown that animals fed upon carbohydrates are in general less susceptible to all those drugs which produce fat accumulation in the liver. We have found that generous feeding of sugar to adult dogs renders them much less susceptible to the late poisonous manifestations of chloroform. For a number of years it has been the custom of many of the surgeons in England, where chloroform is extensively used as a surgical anesthetic, to insure in their patients a good supply of glycogen by generous feeding of carbohydrates.<sup>8</sup> Recently, Opie and Alford<sup>9</sup> have shown that in mice the feeding of carbohydrates exerts a decidedly protective action against the development of liver necrosis by chloroform. Furthermore, Rosenbaum<sup>10</sup> has shown that after a protracted chloroform narcosis the liver is actually poor in glycogen.

The mechanism of this protective action of glycogen is not clear. Is it a property of unchanged glycogen itself, or is it rather a property of glucose? It is, of course, well known that in the condition of so called acidosis of the type which occurs during fasting, diabetes, and some other conditions, the feeding of alcohol, sugars, and other substances possessing alcohol groups, is accompanied by a marked diminution in the output of the acetone bodies in the urine. Chloroform poisoning is one of those conditions. The question of how sugars and other substances accomplish a diminution of this

spectively. During the twelve days after birth the amount of liver glycogen was found gradually to diminish. It is interesting to note, therefore, that the diminution of resistance of pups against chloroform poisoning, which occurs with increasing age, parallels a diminution in the amount of liver glycogen. Mendel and Leavenworth (Chemical Studies on Growth.—III. The Occurrence of Glycogen in the Embryo Pig, *Am. Jour. Physiol.*, 1907-08, xx, 117) speak of high values for pups as being in contrast to those for other fetal tissues; and they seriously question the truth of the statement that in general fetal tissue is rich in glycogen.

<sup>6</sup> Roger, G. H., Action du foie sur la strychnine, *Arch. de physiol. norm. et path.*, 1892, iv, 24.

<sup>7</sup> Rosenfeld, G., Fettbildung, *Ergebn. d. Physiol.*, 1903, ii, pt. 1, 50.

<sup>8</sup> Beddard, A. P., A Suggestion for Treatment in Delayed Chloroform Poisoning, *Lancet*, 1908, i, 782.

<sup>9</sup> Opie, E. L., and Alford, L. B., The Influence of Diet on Hepatic Necrosis and Toxicity of Chloroform, *Jour. Am. Med. Assn.*, 1914, lxii, 895.

<sup>10</sup> Rosenbaum, F., Untersuchungen über den Kohlehydratbestand des tierischen Organismus nach Vergiftung mit Arsenik, Phosphor, Strychnin, Morphium, Chloroform, *Arch. f. exper. Path. u. Pharmacol.*, 1882, xv, 450.

particular type of acidosis has been investigated by Woodyatt.<sup>11</sup> On the basis of the so called Cannizarro reaction which has been extensively studied by Ciamician and Silber,<sup>12</sup> he suggests that sugars and other so called antiketogenic substances undergo oxidation with a simultaneous reduction of ketones and keto-acids. But even if this does explain how antiketogenesis is effected, it does not explain the protective action against liver necrosis. We can not seriously consider the necrosis to be dependent upon an acidosis in the ordinary sense; for even in fatal cases of diabetes with high grade acidosis there is no extensive liver necrosis. On the other hand, it is possible that the protective part played by glycogen against chloroform necrosis is more of a physical nature, accomplished perhaps by altering the permeability of the cell or certain parts of the cell to the chloroform. In this connection it has been shown by Bechhold and Ziegler<sup>13</sup> that glucose, alcohol, and glycerin retard the diffusion of some substances into gels. The protective action ascribed by Whipple to the fetal blood islands found in the livers of these young pups has no evidence to support it, except that blood islands are present in the liver of the insusceptible pups; and, moreover, we have found limited areas of necrosis in livers which still contain some of these blood islands, as shown by the presence of an occasional giant-cell and small collections here and there of mononuclear leucocytes. We shall discuss other means of inhibiting the production of this necrosis in another article, now in preparation, in which evidence will be brought to show that chloroform liver necrosis is produced by hydrochloric acid, which is formed as a dissociation product of chloroform, according to the equation:



A number of experimental difficulties were encountered in carrying on the present work. It was found, for example, that it was almost impossible to maintain an even depth of narcosis in the very

<sup>11</sup> Woodyatt, R. T., The Action of Glycol Aldehyd and Glycerin Aldehyd in Diabetes Mellitus and the Nature of Antiketogenesis, *Jour. Am. Med. Assn.*, 1910, lv, 2109.

<sup>12</sup> Ciamician, G., and Silber, P., Chemische Lichtwirkungen, *Ber. d. deutsch. chem. Gesellsch.*, 1901, ii, 1530, 2040; 1902, ii, 1992; iii, 3593; 1903, ii, 1575.

<sup>13</sup> Bechhold, H., and Ziegler, J., Die Beeinflussbarkeit der Diffusion in Gallerten, *Ztschr. f. physikal. Chem.*, 1906, lvi, 105.



young pups; for when the chloroform was given by inhalation they frequently held their breath for periods of from forty seconds to over a minute. Indeed, in one experiment one of these periods of apnea was found to be as long as two minutes and twenty seconds, during which time the pup lay still and appeared to be deeply anesthetized, but in reality it was entirely conscious and reacted vigorously to stimulation by pinching of the skin, etc. To obviate this error regular artificial breathing was usually maintained by alternate compression and relaxation of the thorax. Because of a scarcity of material, the risk of killing pups prematurely by giving the chloroform by injection was not undertaken. Another difficulty was to avoid death from exposure, since in order to shut off the carbohydrate supply, the pups had to be kept away from their mothers. Still another difficulty was in properly gauging the amount of phlorhizin, so that a two hour chloroform anesthesia would not result in the death of the pup during the next few hours. There was no uniformity in tolerance to the phlorhizin, so that this procedure was largely guess-work.

The chloroform used was Mallinckrodt's, bearing the label "Purified for anesthesia." Usually an anesthesia was maintained for two hours, but sometimes for a longer period. After an interval of two days the pups were killed with chloroform and examined. Sections of the liver and other viscera were stained with hematoxylin and eosin, and also with special fat stains, such as Sudan III. After the pups were taken from their mothers they were kept wrapped in cotton, and artificial heat was supplied so that the temperature of the surrounding air in the box was about 30° C. Water was given daily by subcutaneous injection of 20 to 30 cubic centimeters of 0.85 sodium chloride solution. The phlorhizin was injected subcutaneously, sometimes dissolved in warm 1 per cent. sodium carbonate solution, and at other times suspended in olive oil, according to Coolen's method. Typical protocols follow.

#### STARVATION PRIOR TO ADMINISTRATION OF CHLOROFORM.

*Experiment 5.*—Mar. 10. Three healthy fox-terrier pups were received into the laboratory, all of the same litter, and five days old. Their respective weights were: A, 237 gm.; B, 275 gm.; C, 350 gm.

Mar. 11. After twenty-four hours of starvation A was given chloroform for two hours, and C for three hours.

Mar. 13. All three pups had lost markedly in weight. The respective weights now were: A, 192 gm.; B, 217 gm.; C, 285 gm. Pup B was given chloroform for two hours. Pups A and C were killed with sudden overwhelming doses of chloroform and autopsied. A shows a moderate amount of fat in the liver with virtual absence of subcutaneous and omental fat. Microscopically the liver shows very marked changes. About the central veins are areas involving roughly one-quarter to one-third of the lobule, in which there is marked necrosis, as indicated by fragmentation of nuclei, hyaline changes in the cells, and deep staining of the necrotic part with eosin. Both here and at the periphery of the lobule the cells are very fatty. Pup C has an excessively fatty liver. There are no important changes in the other organs. Microscopically the liver changes are about the same in kind and degree as those in pup A.

Mar. 14. Pup B was again given chloroform for one hour. At the close of the anesthesia it was cold and in very poor condition; respiration was shallow and infrequent. It was kept all night in an incubator at 35° C.

Mar. 15. Pup B was found in the morning to be in much better condition than in the evening before. Killed with chloroform and examined. Weight, 210 gm. There is marked emaciation with an entire absence of omental and subcutaneous fat; the liver is light brown in color. It is everywhere studded with pinhead-sized areas which are pale and opaque and apparently surround the central veins. Microscopically the liver is found to present marked necrosis and to be intensely fatty. The areas of necrosis are central and involve from one-third to one-half of the liver lobules; they are filled with hyaline areas from which the nuclei have disappeared.

#### PHLORHIZIN PRIOR TO ANESTHESIA.

*Experiment 11.*—May 3. Two healthy mongrel pups, two days old, designated as A and B. The respective weights were: A, 390 gm.; B, 370 gm. An injection of 0.05 gm. of phlorhizin was given subcutaneously to A at 9.30 A. M., and repeated at 5 P. M.

May 4. A similar injection of phlorhizin was given again to A at 9.30 A. M. From 1 to 3.30 P. M. both pups were given chloroform. A (phlorhizin pup) took the anesthetic badly and required artificial respiration a number of times. At the close of the anesthesia it was cold and apparently almost dead; both pups were kept in an incubator at about 30° C. all night. We have repeatedly noticed that phlorhizinized animals are much more easily killed with chloroform than are others, a fact which has also been observed by Sansum and Woodyatt<sup>14</sup> in this laboratory. The latter, who made metabolism studies of narcotized diabetic dogs, noted also a great depression of all urinary secretion following the chloroform narcosis.

May 5. B was lively, but A (phlorhizin pup) had shallow respirations and moved only when aroused. At 8.30 P. M. A was moribund; therefore both were killed with chloroform and immediately autopsied. A's (phlorhizin pup) liver was very fatty, B's only moderately so. Microscopically A showed well marked central necrosis involving about one-third of the lobule. B's liver had only a slight amount of necrosis. Both livers contained a few bone marrow cells.

<sup>14</sup> Sansum, W. D., and Woodyatt, R. T., *Jour. Biol. Chem.*, 1915 (in press).

## DETERMINATION OF GLYCOGEN.

May 29. Bulldog pup born last night in the laboratory. At 10.30 A. M. it was taken from its mother. At 2.25 P. M. it was killed by decapitation. The liver was immediately removed and ground in a meat grinder. To the hash, which weighed 9.709 gm., were added 10 c.c. of hot 60 per cent. KOH, and the whole was then placed on a steam bath. This and subsequent operations were carried out in accordance with Pflüger's<sup>15</sup> method. After hydrolysis, 0.95 gm. of glucose, which is equivalent to 9.07 per cent. in 9.709 gm. of liver, was found by titration. The unhydrolyzed glycogen was also determined by means of the polariscope. By this method a value of 9.39 per cent. was obtained, which is seen to be in close agreement with that found by titration after hydrolysis.

## GLUCOSE AND CHLOROFORM.

Mar. 27. Two small adult dogs, designated as A and B. At 10 A. M. A received 15 gm. of glucose in water by stomach tube. From 11 A. M. to 1 P. M. both dogs were given chloroform. At 1 P. M., while still unconscious, A received 25 gm. of glucose in water by stomach tube.

Mar. 28. A was given 50 gm. of glucose by stomach tube.

Mar. 29. Both dogs were killed with chloroform.

*Autopsy.*—A's (glucose dog) liver shows a slight amount of infiltrated fat. Microscopically there is no necrosis. B's liver is intensely fatty; and microscopically there is well marked necrosis. The amount of subcutaneous and omental fat was about equal in the two dogs.

This experiment was repeated on two other sets of dogs, and similar results were obtained.

## SUMMARY AND CONCLUSIONS.

The relative difficulty with which the characteristic central lobular liver necrosis can be produced in young pups after chloroform administration is in some way referable to the high glycogen contents of their livers. Evidence for this conclusion lies in the following facts:

1. Pups can readily be made to show the central liver necrosis which is found in chloroform poisoning in adults, if, prior to the administration of chloroform, they have been starved or starved and made diabetic by phlorhizin.

2. A single quantitative experiment showed that the liver of a normal, well nourished pup, twenty-four hours old, contained as much as 9.07 per cent. of glycogen.

3. The feeding of carbohydrates to adult animals lessens their susceptibility to the production of liver necrosis by chloroform.

<sup>15</sup> Grube, K., *Quantitative Analyse des Glykogens*, in Abderhalden, E., *Handbuch der biochemischen Arbeitsmethoden*, Berlin, 1910, ii, 164.



THE RELATION BETWEEN THE STRUCTURE OF THE  
EPIDERMIS OF THE RAT AND THE GUINEA PIG,  
AND THE PROLIFERATIVE POWER OF NORMAL  
AND REGENERATING EPITHELIAL CELLS  
OF THE SAME SPECIES.\*

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On the basis of earlier studies on the regeneration and transplantation of the skin in various species of animals,<sup>1</sup> Loeb concluded that a definite relation might exist between the structure of the normal skin and its proliferative power. He suggested that an epidermis consisting of a larger number of cell layers, as in the guinea pig, might show under normal and abnormal conditions a greater proliferative power than an epidermis consisting only of one or two cell layers, as in the pigeon; and he believed that the difference in structure between these two types might be due to differences in the proliferative power of the epidermis. Addison and Loeb confirmed the validity of this suggestion.

In accordance with the structure of normal skin, the normal and regenerating epidermis of the pigeon showed less proliferative power than the epidermis of the guinea pig.<sup>2</sup> It was, however, possible that some special condition existed in the pigeon which was responsible for the diminution of proliferative energy in this animal. Before it could be concluded that the relation between the structure and proliferative power of guinea pig and pigeon skin was causal and not accidental, and was of general significance, it was necessary to compare with guinea pig skin, as representative of an epidermis with a larger number of cell layers and correspondingly

\* Received for publication, October 28, 1914.

<sup>1</sup> Loeb, L., and Addison, W. H. F., *Arch. f. Entwicklgsmechn. d. Organ.*, 1911, xxxii, 44.

<sup>2</sup> Loeb and Addison, *Arch. f. Entwicklgsmechn. d. Organ.*, 1913, xxxvii, 635.

greater proliferative power, the skin of another animal possessing an epidermis similar in structure to that of the pigeon.

At the suggestion of Dr. Loeb we made a comparative study of guinea pig and rat skin. The rat was chosen because its epidermis has, like that of the pigeon, only one to two cell layers.

#### NORMAL SKIN.

We studied also normal guinea pig skin and confirmed the figures given in the former paper.<sup>3</sup> The structure of normal guinea pig

TABLE I.  
*Normal Skin.*

	Guinea pig.	Rat.
No. of layers of living cells in the epidermis. . .	3-5	1-2
Thickness of the stratum germinativum. . . . .	25.0-50.0 $\mu$ (25.0-40.0 $\mu$ )	9.6-13.4 $\mu$
Thickness of the upper layer of the epidermis.	20.0-40.0 $\mu$	5.7 - 7.9 $\mu$
Size of cell in basal row		
1 = length. . . . .	10.5 -12.75 $\mu$	7.8 - 9.9 $\mu$
2 = breadth. . . . .	6.0 - 7.0 $\mu$ (9.91-12.8 $\mu$ )	6.0 - 7.0 $\mu$
Size of nucleus in basal row		
1 = length. . . . .	8.5 $\mu$	5.1 - 6.2 $\mu$
2 = breadth. . . . .	5.6 $\mu$ (7.8-8.6 $\mu$ ) (5.6-6.8 $\mu$ )	5.0 - 6.0 $\mu$
No. of mitoses in area 1 mm. long and 50 $\mu$ wide	10-15 (Average, 12) 10-14 (Average, 12)	3-6 (Average, 4.)
No. of cells in basal row in 1 mm. . . . .	155	144

and rat skin is represented in table I; in the case of the guinea pig our new figures are added in parenthesis.

A comparison of this table with table 1 of our previous paper<sup>4</sup> shows the similarity between rat and pigeon skin, which is brought out clearly in almost all the measurements. There is, however, a slight difference in so far as the height

<sup>3</sup>Loeb and Addison, *Arch. f. Entwicklungsmechn. d. Organ.*, 1913, xxxvii, 635.

<sup>4</sup>For the figures referring to the pigeon see Loeb and Addison, *Arch. f. Entwicklungsmechn. d. Organ.*, 1913, xxxvii, 636, 637 (table 1).

of the basal cell and its nucleus is concerned. It appears to be slightly larger in rat than in pigeon skin. The number of mitoses is also slightly greater and the stratum germinativum somewhat thicker in the rat than in the pigeon. The differences between both kinds of epidermis are therefore very slight. In the guinea pig the stratum germinativum is almost three times as thick as in the pigeon or rat. The same unit area for counting mitoses was used as in the former work; namely, an area 1 mm. long and  $50\mu$  wide. In the rat 3 to 6, on the average 4, mitoses were found in that area. In the pigeon the average was 3, while it was 12 in the guinea pig. There are approximately 1,204 cells in the unit area in the guinea pig with an average of 12 mitoses; almost all mitoses are found in the basal layer. There occurs then about 1 mitosis in every 100 basal cells in the epidermis of the guinea pig. In the rat we find in the same unit area approximately 1,036 cells. There occurs 1 mitosis in every 259 basal cells in the epidermis of the rat.

There are, therefore, 2.5 times as many mitoses in the same number of cells in the guinea pig as in the rat. The potential energy displayed in the basal cells of the guinea pig is therefore almost 2.5 times as large as in the rat.

#### REGENERATING SKIN.

In table II will be found the figures representing the conditions in the regenerating epidermis of the rat. In the case of the guinea pig and pigeon we refer to the corresponding tables of the former paper.<sup>5</sup>

The wound made in the rat skin was of the same length as in the guinea pig and pigeon; namely, approximately 3 mm. in both diameters. All observations and measurements were made in exactly the same manner as in the previous paper, with the same stages of regeneration and the same places in the wound. Before the closure of the wound the number of living cell rows and the thickness of the living epithelium were measured at the tip of the tongue, at the juncture of the tongue with the old epithelium, and in the old epithelium near the insertion of the tongue. After closure of the wound, measurements were taken at two places; namely, in the center of the former defect and at the insertion of the former tongue.

Two days after excision of the piece of skin, the size of the regenerating epithelial tongue in the guinea pig is much greater than in the pigeon and rat. In the rat it is slightly larger than in the pigeon; it grows in the guinea pig more rapidly than in either of the two other animals. The defect is greater in the pigeon than in the rat and guinea pig; in these two animals it is very similar. This is probably due to the fact that in the pigeon a retraction of the margin of the wound usually takes place, while such a retraction does not occur in the guinea pig and rat. In the rat the tongue grows constantly up to the ninth day, but towards the end of this period near the time of closure of the wound the increase is very slight. Also, in the other animals there is a

<sup>5</sup> Loeb and Addison, *Arch. f. Entwicklungsmechn. d. Organ.*, 1913, xxxvii, 641, 642, 648, 649 (tables 2, 3, 4, 5).

TABLE II.  
*Rat Skin.*

Dy.	No. of pieces of skin.	Length of tongue.	Length of defect.	Length of tongue and defect.	No. of rows of living cells and thickness of stratum germinativum.	Old epithelium.
2	1	0.006 mm.	2.907 mm.	3.093 mm.	Tip of tongue.	
	2	0.088-0.094 mm.	2.001 mm.	2.183 mm.	1-2 cell rows, 5.7 $\mu$	1-2 cell rows, 11.5 $\mu$
	3	0.153 mm.	2.769 mm.	3.068 mm.	1-2 cell rows, 7.6 $\mu$	1-2 cell rows, 9.6 $\mu$
5	1	0.266 mm.	1.922 mm.	2.777 mm.	1-2 cell rows, 6.9 $\mu$	1-2 cell rows, 11.3 $\mu$
	2	0.359 mm.	1.710 mm.	2.251 mm.	2 cell rows, 13.49 $\mu$	1-2 cell rows, 13.4 $\mu$
	3	0.317 mm.	2.571 mm.	3.363 mm.	2 cell rows, 15.53 $\mu$	1-2 cell rows, 15.1 $\mu$
7	1	0.538 mm.	0.349 mm.	2.122 mm.	2 cell rows, 13.53 $\mu$	1-2 cell rows, 14.6 $\mu$
	2	0.407 mm.	0.203 mm.	1.846 mm.	2 cell rows, 13.4 $\mu$	1-2 cell rows, 17.6 $\mu$
	3	0.459 mm.	0.145 mm.	2.031 mm.	2 cell rows, 11.9 $\mu$	1-2 cell rows, 15.3 $\mu$
9	1	0.649 mm.	0.146 mm.	2.196 mm.	2 cell rows, 12.1 $\mu$	1-2 cell rows, 11.9 $\mu$
	2	0.593 mm.	0.115 mm.	1.257 mm.	2 cell rows, 13.5 $\mu$	1-2 cell rows, 12.7 $\mu$
	3	Wound closing			2 cell rows, 12.4 $\mu$	1-2 cell rows, 14.6 $\mu$
11	1		Closed		4-5 cell rows, 46.1 $\mu$	1-2 cell rows, 11.5 $\mu$
	2		Closed		3-4 cell rows, 39.6 $\mu$	1-2 cell rows, 11.8 $\mu$
	3		Closed		5 cell rows, 49.9 $\mu$	1-2 cell rows, 10.7 $\mu$
14	1		Closed		Insertion of former tongue	1-2 cell rows, 13.5 $\mu$
	2		Closed		4 cell rows, 43.1 $\mu$	1-2 cell rows, 10.1 $\mu$
	3		Closed		4-5 cell rows, 47.2 $\mu$	1-2 cell rows, 11.5 $\mu$



constant growth of the tongue up to the time of the closure, but the rate of growth is not identical in the three species.

On the 2d day the tongue is greatest in the guinea pig; on the 5th day it reaches in all animals about three to four times the size it had on the 2d day. The increase is, however, greatest in the guinea pig, and least in the pigeon. Between the 5th and 7th days, the increase is relatively less in the guinea pig than in the pigeon and rat. Between the 7th and 9th days the increase in the tongue is relatively less than in the previous periods. We therefore notice in all species a gradual diminution in the relative increase of the tongues (expressed in percentage increase over the size at the end of the preceding period) after the 2d day.

The closure of the wound in the rat as well as in the other animals does not take place merely through an outgrowth of the tongue, but also through a contraction of the wound as a whole.

The number of rows of living cells and the thickness of the living epithelium varies at different periods of regeneration. We find the count in the pigeon and rat to be nearly the same, the figures being usually a little greater in the rat than in the pigeon, but they are much greater in the guinea pig than in the pigeon or rat. We find some differences according to the various places where measurements were taken. In the pigeon and rat the thickness of the living epithelium at the tip of the tongue and in the old epithelium are very similar, while at the point of insertion the tongue is much thicker than at either of the other two places. In the guinea pig the old epithelium is, at the various stages of regeneration, usually thicker than the epithelium at the tip of the tongue. As in the rat and pigeon, in the guinea pig the epithelium is thickest at the point of insertion of the tongue. In the guinea pig the greatest thickness of the regenerating epithelium at the point of insertion of the tongue is reached between the 7th and 11th days, while in the old epithelium the greatest thickness is reached on the 7th day at the time of closure. In the pigeon the greatest thickness is reached between the 5th and 11th days, but here again at the point of insertion of the tongue the epithelium is thickest just previous to the time of closure on the 9th day; while in the rat the thickness is greatest from the 5th to the 14th day. As soon as the tips of the tongues meet, the thickness of the living epithelium in the middle of the former wound is greater than it was previously at the tip of the tongue in the rat and pigeon. This indicates that the two tongues in meeting exert pressure upon each other. The wound closes on the 7th day or soon afterwards in the guinea pig, on the 11th day in the pigeon, and between the 9th and 10th days in the rat. There is, therefore, no marked connection between the thickness of the regenerating epithelium and the time of closure of the wound, other than as indicative that at some place the regenerating epithelium is slightly thicker just before or at the time of the closure, and that the closure effects the thickness in the center of the wound, the thickness at this place after the closure being greater than at the tip of the former tongue. In the guinea pig the maximum of the thickness of the living epithelium is about twice as large at the point of the insertion of the tongue and about four times as large in the old epithelium as in the rat and in the pigeon.

The figures in table III represent the size of cell and nucleus and the number of mitoses during the different stages of regeneration in the rat. For the

TABLE III.

*Rat Skin.*

Dy.	No. of pieces of skin.	Size of cell.	Size of nucleus.	No. of mitoses in one area 1 mm. long $\times$ 50 $\mu$ wide.	
				New epithelium.	Old epithelium.
2	1	86.5 $\mu$ 11.5 $\times$ 7.6 $\mu$	48.3 $\mu$ 7.6 $\times$ 6.1 $\mu$	0	24
	2	11.3 $\times$ 7.9 $\mu$	8.0 $\times$ 7.9 $\mu$	0	26
	3	10.9 $\times$ 7.6 $\mu$	8.0 $\times$ 5.9 $\mu$	0	28
5	1	96.8 $\mu$ 11.7 $\times$ 7.9 $\mu$	55.8 $\mu$ 8.8 $\times$ 6.7 $\mu$	23	22
	2	12.2 $\times$ 8.0 $\mu$	8.1 $\times$ 6.5 $\mu$	28	23
	3	12.6 $\times$ 8.1 $\mu$	8.8 $\times$ 6.1 $\mu$	17	17
7	1	111.3 $\mu$ 13.6 $\times$ 9.0 $\mu$	64.2 $\mu$ 9.3 $\times$ 6.9 $\mu$	27	16
	2	12.7 $\times$ 8.6 $\mu$	8.8 $\times$ 6.1 $\mu$	31	13
	3	13.2 $\times$ 8.1 $\mu$	10.3 $\times$ 7.6 $\mu$	42	11
9	1	102.8 $\mu$ 12.1 $\times$ 9.0 $\mu$	59.4 $\mu$ 8.3 $\times$ 6.6 $\mu$	23	9
	2	12.9 $\times$ 7.9 $\mu$	9.0 $\times$ 7.2 $\mu$	15	9
	3	11.3 $\times$ 8.7 $\mu$	8.8 $\times$ 7.0 $\mu$	31	15
11	1	86.9 $\mu$ 10.2 $\times$ 6.9 $\mu$	47.5 $\mu$ 7.6 $\times$ 6.1 $\mu$	12	8
	2	11.1 $\times$ 8.8 $\mu$	7.2 $\times$ 6.5 $\mu$	15	10
	3	11.8 $\times$ 8.0 $\mu$	6.7 $\times$ 5.9 $\mu$	10	9
14	1	65.4 $\mu$ 8.9 $\times$ 6.9 $\mu$	42.48 $\mu$ 7.1 $\times$ 6.1 $\mu$	15	7
	2	9.2 $\times$ 7.3 $\mu$	6.6 $\times$ 5.8 $\mu$	9	6
	3	10.2 $\times$ 7.3 $\mu$	6.9 $\times$ 5.9 $\mu$	11	6

corresponding figures in the guinea pig and pigeon the reader is referred to the former paper.<sup>6</sup> In the guinea pig the cell increases in size up to the 7th day when the maximum size is reached. This is followed by a sudden decrease which makes the cell slightly smaller than it had been on the 2d day, but it remains somewhat larger than in the normal skin throughout the period of observation. The changes in the nucleus are similar to those in the whole cell. The size of the nucleus also reaches a maximum on the 7th day, shows a marked decrease on the 9th day, but remains larger than the nucleus of the normal skin throughout observation. The number of mitoses also reaches a maximum on the 7th day and experiences a sudden sharp fall on the 9th, and declines very slowly but steadily from the 9th to the 14th day. The number on the 14th day is still higher than in the normal skin.

The time of the maximum size of cell and nucleus and the time of the maximum number of mitoses correspond very well with each other, and both coincide with the time just preceding the closure of the wound. The closure of the wound is followed by a marked fall in the figures representing the size of cell and nucleus and the number of mitoses.

<sup>6</sup> Loeb and Addison, *Arch. f. Entwicklungsmechn. d. Organ.*, 1913, xxxvii, 635.

In the pigeon the maximum size of cell and nucleus is also reached on the 7th day. On the 9th day we notice a decline in the size of cell and nucleus, which, however, remain increased throughout the period of observation. The maximum number of mitoses is reached between the 5th and 9th days; it is followed by a marked decline on the 11th day, but the number remains increased over the number in the normal epidermis throughout the period of observation. We see, therefore, that here the maximum in the number of mitoses, while reached as early as in the guinea pig (or even slightly earlier), extends over a longer period; this increase in duration is probably connected with the later closing of the wound in the pigeon. There is, however, in the pigeon a slight discrepancy between the curves of cell size and of number of mitoses, in as far as here the curve of the size of cell and nucleus begins to decline on the 9th day. The decline in the cell size on the 9th day is, however, relatively slight. The maximum for the number of mitoses and size of cell is present in the pigeon as early as on the 7th day, although the wound does not close for 3 or 4 days later.

In the rat the maximum size of cell and nucleus is reached on the 7th day; the size of cell and nucleus declines very slightly on the 9th day. It continues to decline steadily from then to the 14th day, but is still larger at that time than in the normal skin. The maximum number of mitoses is reached on the 7th day, but it is almost as high on the 5th as on the 7th day. There is a decrease from the 7th day to the 9th day, then there occurs a further decided decrease on the 11th day, and the number continues to decrease on the 14th day, but it is still higher at that time than in the normal rat skin.

We see in every case a marked decline in the number of mitoses at the time of closure of the wound, and the maximum number is reached on the 7th day, or even shortly before. There is a constant increase from the beginning to the 7th day, and while it is already high at the 5th day in the three species, in most cases it is not quite as great at that time as on the 7th day. The decline after the closure of the wound is sharpest in the guinea pig; here the difference between the maximum number of mitoses on the 7th day and the number on the 11th and 14th days is greater than in the pigeon and rat.

We find, therefore, in all three species during the regeneration of the epidermis the maximum in size of cells and nuclei approximately on the 7th day, independently of the closure of the wound. In case the wound is not yet closed on the 7th day, the size of the cell and the number of mitoses either do not decline, or they decline relatively less until the closure takes place, when a more marked decline occurs. The size of cells and nuclei and the number of mitoses remain increased over the normal figure throughout the period of observation.

We now wish to consider how the three species differ among themselves in the figures for the size of cell and nucleus and for the number of mitoses. During the process of regeneration the size of the guinea pig cell is the greatest. In the rat almost the same figures are found as in the pigeon, but the figures in the rat are slightly higher than those in the pigeon. In both these animals the figures are markedly lower than in the guinea pig. Mitotic cell proliferation and the size of cells and nuclei show, therefore, proportionately the same differences in the regenerating tissue of these three species as in the normal skin.

The relative increase in mitoses during regeneration over the number found in the normal epidermis is apparently not quite so large in the guinea pig as in

the rat and pigeon. The normal epidermis of the guinea pig has in the same area four times as many mitoses as the normal epidermis of the pigeon, but during regeneration it has only 2 to 2.5 times as many mitoses as the regenerating epidermis of the pigeon. The normal epidermis of the guinea pig has about three times as many mitoses as the normal epidermis of the rat, while in the regenerating epithelium the number of mitoses in the guinea pig is less than three times as high as in the rat, if we compare during regeneration each time the maximum number of mitoses. At other periods the superiority in the number of mitoses on the part of the guinea pig is still less marked. We see, therefore, that apparently the effect of a regenerative stimulus is relatively not quite so great in skin in which under normal conditions much proliferation is taking place (guinea pig) as in skin in which under normal conditions less proliferation is taking place (rat). This method of calculating, however, does not consider the difference in the number of cells in the same area in the different species of animals. We must consider the number of mitoses in each species in proportion to the number of cells in a certain area in the resting and in the regenerating epithelium of the various animals.

In normal guinea pig epidermis we found 1 mitosis to every 100 cells; in normal rat skin 1 mitosis to every 250 cells. In guinea pig skin, therefore, there are in the same number of cells 2.5 times as many mitoses as in the rat. In the regenerating skin the size of the cell increases. While in all the species the size of the regenerating cell is greater than the resting cell, the proportional increase in the size of the regenerating cell over the resting cell is approximately the same in the guinea pig, pigeon, and rat, or if any difference exists it is only slight. We may, therefore, consider the number of cells upon which we base our comparisons of the increase in the number of mitoses during regeneration in the rat and guinea pig as approximately constant; because the number of changes in both species has approximately the same proportion, the size of the regenerating cell in the guinea pig, as well as in the rat, being approximately four to five times larger than the resting cell of the same species, if we use two diameters in calculating the size of the cells. If we consider, therefore, the number of mitoses in both species occurring in the same number of cells under normal conditions and during regeneration, the increase during regeneration is approximately as great in the guinea pig as in the rat. We may, therefore, conclude that the proliferative energy of the epithelial cell of the guinea pig is stimulated through making a wound just as effectively as the epithelial cell of the rat. The effect of stimulation as evidenced in the increase in proliferation during regeneration is as great in the guinea pig as in the rat.

A comparison of the number of mitoses in the different species at the different periods of regeneration shows that after the closure of the wound the difference in the number of mitoses between guinea pig skin on the one hand and rat and pigeon skin on the other is much less than before the closure of the wound, which is another expression of the fact that the decline in the number of mitoses after the closure of the wound in the guinea pig is much greater than in the rat and pigeon.

A comparison of the relative number of mitoses in the different parts of the epithelium during regeneration shows the following: Two days after the making of the wound all the mitoses are found in the old epithelium and none are

observed in the epidermal tongue. This applies to all three species. After 5 days the number of mitoses in the tongue is somewhat larger than in the old epithelium in the guinea pig. The same holds good in the pigeon, while the number is about equal in both places in the rat. On the 7th day in all three species the number of mitoses is much greater in the new than in the old epithelium. After 9 days the number of mitoses is greater in the old than in the new epithelium in the guinea pig, while in the rat and pigeon the number of mitoses is still greater in the new epithelium, because in the two latter animals the wound is either not yet closed or is just at the point of closing at this period, in contradistinction to the guinea pig, where the closure has taken place previously. On the 11th and 14th days the difference between the number of mitoses in the old and new epithelium is very slight in all three species. The number is, however, in the rat and in the pigeon slightly greater in the new than in the old epithelium, while it is about the same in the guinea pig.

We find, therefore, in all three species the increase in mitoses at first entirely confined to the old epithelium. Then the number of mitoses becomes greater in the new than in the old epithelium on the 7th day. After closure of the wound on the 11th and 14th days the number of mitoses is slightly greater in the new than in the old epithelium in the case of the pigeon and rat, while it is about equal at both places in the guinea pig.

#### SUMMARY.

1. A comparative study of the resting and regenerating epidermis of the rat, pigeon, and guinea pig shows the connection between the structure of the skin and the proliferating power of the epithelial cells. We may at present distinguish two types of epidermal cells: (a) cells with less proliferative energy (pigeon and rat), and (b) cells with a greater proliferative power (guinea pig); the former provides the element from which the type of a thin epidermis is built up, while from the latter the type of a thick epidermis develops.

2. The cell type with greater proliferative power in the normal skin shows also greater proliferative power during regeneration, and the regenerative stimulus causes approximately the same relative increase in the number and size of cells and nuclei in both kinds of skin during regeneration, provided that we base our determination of the number of mitoses on the same unit number of cells in both skins rather than on the same unit area. The absolute increase in the number of cells during regeneration is greatest in the type with the greatest proliferative power in the normal cell (guinea pig).

3. The number of mitoses and the size of cells and nuclei follow a

similar curve in the different species during regeneration. They reach a maximum approximately on the 7th day; remain stationary or show some decline from the 7th day on until the time of the closure of the wound; following the closure a further fall takes place; but the figures remain higher than in the normal epidermis for a considerable period afterwards. There seems, therefore, to exist under the conditions of our experimentation a period of approximately 7 days, at the end of which the regenerative stimulus reaches its maximum effect.

The closure of the wound represents probably an additional factor determining the curve of cell proliferation. Failure of the wound to close at an early date seems to make the decline in the number of mitoses sharp after the maximum has been reached.

4. The curve representing the thickness of living regenerating epithelium declines much more slowly than the curve representing cell size and number of mitoses. This indicates probably that even after closure of the wound some push of epithelial cell towards the center of the former wound continues to take place.

5. From the second day the additional growth of the epidermal tongue decreases in each successive period, if we use the length of the tongue at the beginning of each period as the unit of measurement.

6. Retraction and contraction of the wound are factors additional to epithelial movements and epithelial proliferation determining the closure of the wound.

## LIVER FUNCTION AS INFLUENCED BY ANESTHETICS AND NARCOTICS.\*

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The functional capacity of the liver may be estimated by means of phenoltetrachlorphthalein. This drug when given intravenously in a normal dog will be excreted solely in the bile and can be recovered from the feces. The output of normal dogs is fairly constant, and the upper and lower limits of excretion may be placed at 65 and 45 per cent., respectively. A normal dog under uniform conditions gives a constant output and rarely varies more than 10 per cent. between the limits of 65 and 45 per cent.

In earlier publications<sup>1,2</sup> we have reported experiments to show that phenoltetrachlorphthalein is removed from the body fluids by the activity of the hepatic epithelium and escapes with the bile into the intestinal tract. Any poison which injures the hepatic epithelium (chloroform, phosphorus, hydrazin) will cause a drop in the output of phthalein, and this fall in the phthalein curve will be proportional to the amount of liver injury. In severe liver injury the phthalein output may fall to zero. Injury of the liver by actual cautery will cause a fall in the phthalein output, depending upon the tissue destroyed and the inflammatory reaction. Given an actual injury of the liver cell, it will be noted that the phenoltetrachlorphthalein appears in the urine in demonstrable quantities, even 1 to 10 per cent. This is never demonstrated in normal animals.

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<sup>1</sup> Whipple, G. H., Mason, V. R., and Peightal, T. C., *Bull. Johns Hopkins Hosp.*, 1913, xxiv, 207.

<sup>2</sup> Whipple, G. H., Peightal, T. C., and Clark, A. H., *Bull. Johns Hopkins Hosp.*, 1913, xxiv, 343.

After a liver injury by means of chloroform the liver may repair its injured cells and form new ones. At this time the phthalein excretion may rise above normal, indicating perhaps that the new cells are more active. The same indication of hyperactivity may be noted after small doses of hepatic poisons, which may have an irritant effect or actually stimulate the liver cells. Hyperactivity is quite distinct in the condition of tetany brought about by removal of the parathyroid glands.<sup>3</sup>

Vascular disturbances may so modify the environment of the liver cell as to cause various degenerations; for example, fatty degeneration in passive congestion. In experimental passive congestion the phthalein output may show a diminution depending upon the severity of the passive congestion. The Eck fistula which shunts the portal blood around the liver and reduces the blood circulating through the liver to about one-third normal, will modify the excretion of phenoltetrachlorphthalein.

The ductless glands modify the functional activity of the liver, as pointed out in a recent publication. Pancreatic extirpation is followed by a falling curve of phthalein excretion which may go as low as one-third normal. This indicates that the pancreas is essential for proper liver function and suggests that the pancreas may have an accelerating influence on the liver.

Adrenal insufficiency is accompanied by a fall in the phthalein excretion, and with hypertrophy of the remaining adrenal fragment the liver excretion returns to normal.

Hypophysis extirpation is followed by minor fluctuations in the phthalein curve with a final drop which takes place shortly before the drop in body temperature which precedes the fatal outcome.

Thyroid removal causes no change in phthalein excretion. Parathyroid extirpation with tetany is associated with a maximum excretion of phenoltetrachlorphthalein, and in many instances evidence of hyperexcretion and abnormal activity of the liver cells. We may assume that in the condition of parathyroid tetany the liver cells are acted on by some powerful stimulus,—that in this condition they remove the phthalein from the blood and pour it into the intestine very promptly. In fact, the liver output will almost

<sup>3</sup> Whipple, G. H., and Christman, P. W., *Jour. Exper. Med.*, 1914, xx, 297.



equal the amount recovered from the feces after mouth feeding, almost a quantitative elimination of the drug through the liver.

All these experiments show how accurate an index of liver activity is the excretion of phenoltetrachlorophthalein. As experimental evidence accumulates we learn to place more and more confidence in this drug as an indicator of hepatic activity. The experiments given below show that ether anesthesia given over a period of two hours may cause interference with liver function lasting over a period of twenty-four hours after the anesthesia. There is no comparison, of course, with the injury done by chloroform anesthesia, which causes actual liver necrosis and much greater impairment of liver function.

Paraldehyde in sufficient amounts to produce stupor usually causes a drop in the phthalein curve. Chloral and urethane give very similar results. Small doses may cause no depression, but rather a maximum output, suggesting that small doses may actually stimulate the liver cells.

Alcohol in large doses sufficient to produce stupor for several hours causes a depression of the phthalein excretion and indicates a definite injury to the liver cells. That the liver at this time might be more susceptible to various injurious agents, for example bacteria, seems highly probable.

When phthalein injection gives evidence of a depression of liver activity to two-thirds or one-half normal, we may assume a very definite interference with liver activity. A depression of this amount may be found in an Eck fistula liver, or in one handicapped by passive congestion, or even in one injured by phosphorus or hydrazin. This comparison shows that the injury done the liver by these anesthetics and narcotics, although temporary, is really definite and worthy of consideration.

#### METHOD.

The method used has been described in a recent publication,<sup>4</sup> and in fact these experiments were done at that time with a great number of control experiments. The references given<sup>5,6,7</sup> cover all the points concerning the prep-

<sup>4</sup> Whipple and Christman, *loc. cit.*

<sup>5</sup> Whipple, Peightal, and Clark, *loc. cit.*

<sup>6</sup> Rowntree, L. G., Hurwitz, S. H., and Bloomfield, A. L., *Bull. Johns Hopkins Hosp.*, 1913, xxiv, 327.

<sup>7</sup> Whipple and Christman, *loc. cit.*

aration of the drug for injection, the extraction of the feces, and the general control and conduct of the experiments.

#### EXPERIMENTAL OBSERVATIONS.

##### ETHER ANESTHESIA.

- Dog 13-1.*—Young mongrel, male; weight 14 pounds.  
 Oct. 23. Phthalein 0.16 gm. intravenously.  
 Oct. 24. Abundant feces. Phthalein excretion 48 per cent.  
 Oct. 30, 11 A. M. Ether anesthesia 2 hours. At the end of the anesthesia phthalein 0.1 gm. given intravenously. Urine shows a trace of phthalein.  
 Oct. 31. Abundant feces. Phthalein excretion 26 per cent.  
 Nov. 6. Phthalein 0.1 gm. intravenously. Urine contains no phthalein.  
 Nov. 8. Formed feces. Phthalein excretion 44 per cent.

##### ETHER ANESTHESIA.

- Dog 12-104.*—Small fox-terrier, male; weight 15¾ pounds.  
 Oct. 30, 1 P. M. Phthalein 0.1 gm. intravenously.  
 Oct. 31. Abundant feces. Phthalein excretion 49 per cent.  
 Jan. 15, 3 P. M. Ether anesthesia 2 hours. At the end of the anesthesia phthalein 0.1 gm. given intravenously.  
 Jan. 17. Abundant feces. Phthalein excretion 39 per cent.

##### ETHER. ALCOHOL. CHLOROFORM.

- Dog 13-55.*—Strong bulldog, male; weight 23 pounds.  
 Jan. 20. Phthalein 0.2 gm. intravenously.  
 Jan. 21. Abundant feces. Phthalein excretion 66 per cent.  
 Feb. 4. Light ether anesthesia 2 hours. At the end of the anesthesia phthalein 0.2 gm. given intravenously.  
 Feb. 5. Abundant feces. Phthalein excretion 63 per cent.  
 Feb. 11. Ether anesthesia 2 hours; deeper anesthesia than before. At the end of the anesthesia 0.2 gm. phthalein given intravenously.  
 Feb. 12. Urine shows a trace of phthalein; no feces.  
 Feb. 14. Abundant feces. Phthalein excretion 53 per cent.  
 Feb. 18, 10 A. M. Dog given 50 c.c. of 95 per cent. alcohol by stomach tube. This caused much intoxication, but dog was able to walk a little. 5 P. M. Dog recovering from intoxication. Phthalein 0.2 gm. intravenously.  
 Feb. 20. Some difficulty with purgation. Phthalein excretion 60 per cent.  
 Apr. 21. Chloroform anesthesia 1 hour.  
 Apr. 22. Phthalein 0.2 gm. intravenously. Urine contained much phthalein (7 per cent.).  
 Apr. 24. Abundant feces. Phthalein excretion 31 per cent.

The preceding experiments show that ether anesthesia for two hours may cause a temporary decrease in hepatic secretion and elimination of phenoltetrachlorophthalein. It is to be kept in mind that the phthalein is injected at the end of the period of anesthesia

and its excretion takes place during the following twenty-four hours. The evidence shows that the injury done by the drug affects the functional capacity of the liver for the twenty-four hours subsequent to the anesthesia. There is no histological evidence of liver injury, but we have shown elsewhere that this physiological test is more sensitive than any estimate based on histological evidence. Ether anesthesia for one hour will scarcely cause any evidence of depressed liver function, and in many instances a two hour light surgical anesthesia will not cause any depression in the phthalein curve of a strong dog.

Ether anesthesia causes a small amount of phthalein to appear in the urine, which speaks in favor of a slight actual injury to the liver parenchyma. It is seen in the preceding experiment that a large amount of phthalein escapes in the urine after a chloroform anesthesia, which is known to cause outspoken central liver necrosis. It has been shown in earlier communications that injury of the liver cell is responsible for a modification of the phenoltetrachlorophthalein, which permits it to pass the kidney filter. We may assume then that there is in all probability a slight injury produced in the liver cells by two hours of ether anesthesia.

## PARALDEHYDE.

*Dog 13-50.*—Young terrier, male; weight 14¾ pounds.

Jan. 17. Dog has distemper, but is in fair condition. 11 A. M. Paraldehyde 6.5 c.c. given by stomach tube. 11.30 A. M. Dog is reeling about cage in wild excitement. 1 P. M. Periods of excitement and again of stupor. Phthalein 0.1 gm. intravenously and paraldehyde 1 c.c. given by stomach tube. 2.30 P. M. Dog much intoxicated and disoriented. 4.30 P. M. Condition unchanged.

Jan. 18, 11 A. M. Dog recovered from drug. Urine shows only a faint trace of phthalein.

Jan. 19. Abundant feces. Phthalein excretion 33 per cent.

Jan. 28. Dog quite sick with distemper and bad diarrhea. Phthalein 0.1 gm. intravenously. Urine shows no phthalein.

Jan. 29. Fluid feces. Phthalein excretion 52 per cent.

## PARALDEHYDE.

*Dog H-2.*—Small mongrel; weight 13½ pounds.

Jan. 8. Phthalein 0.1 gm. intravenously.

Jan. 9. Phthalein excretion 55 per cent.

Jan. 11. Phthalein 0.1 gm. intravenously. Dog has little distemper and weighs only 12½ pounds. Urine contains no phthalein.

## 208 *Liver Function as Influenced by Anesthetics and Narcotics.*

Jan. 12. Abundant feces. Phthalein excretion 54 per cent.

Jan. 20, 12 M. Paraldehyde 5 c.c. by stomach tube. 1 P.M. Anesthesia complete; no period of excitement. Muscular tremors noticeable. 3 P.M. Dog excited and reels about cage. 5 P.M. Dog very noisy and excited, rolls about cage. Phthalein 0.1 gm. intravenously.

Jan. 21. Urine contains much phthalein (0.1 per cent.  $\pm$ ).

Jan. 22. Fluid feces. Phthalein excretion 25 per cent. 3 P.M. Dog given ether and killed.

*Autopsy.*—The organs are all negative except the liver. Bile passages are normal. Liver shows slight central atrophy and a little fatty degeneration at the edge of the liver lobules. Microscopical section shows a little fatty degeneration of the liver cells, but the nuclei are normal and the fat droplets not numerous. The endothelial cells contain a good deal of yellow pigment.

### PARALDEHYDE.

*Dog 13-57.*—Mongrel fox-terrier; weight 11½ pounds.

Jan. 28, 12 M. Phthalein 0.1 gm. intravenously.

Jan. 29. Abundant feces. Phthalein excretion 58 per cent.

Feb. 10. Phthalein 0.1 gm. intravenously.

Feb. 11. Abundant fluid feces. Phthalein excretion 66 per cent.

Mar. 18, 9.30 A.M. Dog is well. Paraldehyde 5½ c.c. with 4 c.c. of alcohol given by stomach tube. 10.30 A.M. Dog is much intoxicated; salivation marked. 11 A.M. Dog not under anesthetic, but rolls about aimlessly. 4 P.M. Dog out of influence of drug. Phthalein 0.1 gm. intravenously. Urine shows no phthalein.

Mar. 19. Abundant fluid feces. Phthalein excretion 65 per cent.

The three preceding experiments indicate that paraldehyde in doses sufficient for anesthesia causes a lowering of the functional capacity of the liver during the following twenty-four hours. A dose which does not cause anesthesia may or may not cause depression of the liver function. The last experiment shows the maximum excretion of phthalein after a dose of paraldehyde sufficient to cause intoxication for four or five hours.

### ALCOHOL.

*Dog 13-16.*—Strong mongrel, male; weight 21¼ pounds.

Nov. 22. Phthalein 0.2 gm. intravenously.

Nov. 24. Abundant feces. Phthalein excretion 56 per cent.

Dec. 20, 11 A.M. Dog given 50 c.c. of 95 per cent. alcohol by stomach tube. 2 P.M. Dog is intoxicated, but can walk with difficulty. 4 P.M. Dog still intoxicated. Phthalein 0.2 gm. intravenously. At the same time the dog is given 20 c.c. of 95 per cent. alcohol.

Dec. 21. No feces. Urine contains no phthalein.

Dec. 22. Abundant feces. Phthalein excretion 55 per cent.

Jan. 20. Dog is in good condition. Weight 24¾ pounds. 12 M. Dog given

50 c.c. of 95 per cent. alcohol by stomach tube; no food given previously. 12.30 P. M. Dog in stupor and unable to walk. 3 P. M. Dog in stupor and can not be roused. 5 P. M. Dog still deeply intoxicated, but attempts to move. Given 25 c.c. of 95 per cent. alcohol. Phthalein 0.2 gm. intravenously. Urine shows no phthalein.

Jan. 21, 11 A. M. Dog is still somewhat intoxicated and weak.

Jan. 22. Abundant feces. Phthalein excretion 38 per cent.

Mar. 25. Dog is well. 10.30 A. M. Dog given 30 c.c. of 95 per cent. alcohol by stomach tube. 11 A. M. Dog is intoxicated and staggers about cage. 12.30 A. M. Dog is restless and excited. Phthalein 0.2 gm. intravenously. 3 P. M. Recovery almost complete.

Mar. 27. Abundant feces. Phthalein excretion 54 per cent.

#### ALCOHOL.

*Dog 13-29.*—Strong bulldog, female; weight 27 pounds.

Dec. 27. Phthalein 0.2 gm. intravenously.

Dec. 29-30. Purgation delayed. Phthalein excretion 48 per cent.

Jan. 7. Phthalein 0.2 gm. intravenously.

Jan. 8. Abundant feces. Phthalein excretion 45 per cent.

Mar. 18, 9.30 A. M. Dog given 50 c.c. of 95 per cent. alcohol by stomach tube. 10.30 A. M. Dog vomited some fluid and alcohol. 10.45 A. M. Dog is drowsy, but not intoxicated. Given 30 c.c. of 95 per cent. alcohol. 11.30 A. M. Dog deeply intoxicated. 4 P. M. Dog still much intoxicated. Given 25 c.c. of 95 per cent. alcohol. Phthalein 0.2 gm. intravenously.

Mar. 19. Dog still somewhat intoxicated and thirsty.

Mar. 20. Abundant feces. Phthalein excretion 28 per cent.

The three preceding experiments show that large doses of alcohol sufficient to cause stupor for a few hours may bring about a decreased phthalein excretion during the twenty-four hours following administration of the drug. Some animals are much more resistant than others and may take large doses without any demonstrable variation in the phthalein curve.

#### ALCOHOL. URETHANE.

*Dog 13-25.*—Strong brindle bull, male; weight 21 pounds.

Dec. 18. Phthalein 0.2 gm. intravenously.

Dec. 19. Abundant feces. Phthalein excretion 65 per cent.

Jan. 10. Phthalein 0.2 gm. intravenously.

Jan. 11-12. Little delay in purgation. Phthalein excretion 55 per cent.

Jan. 13, 12 M. Dog given 50 c.c. of 95 per cent. alcohol by stomach tube. 2.30 P. M. Dog pretty much intoxicated. Given 40 c.c. of 95 per cent. alcohol. 3.30 P. M. Dog is deeply intoxicated and lies on side. 4.30 P. M. Vomits fluid and food. 6 P. M. Phthalein 0.2 gm. intravenously. Jan. 15. Abundant feces. Phthalein excretion 44 per cent.

## 210 *Liver Function as Influenced by Anesthetics and Narcotics.*

Mar. 25, 10.30 A. M. Dog is well; weight 27 pounds. Given 12 gm. of urethane in water by stomach tube. 11 A. M. Dog excited and intoxicated, but can walk about. 12.30 P. M. Dog intoxicated, but conscious. Given 4 gm. of urethane. Phthalein 0.2 gm. intravenously. 5 P. M. Dog reels about, but is quite active,—not drowsy.

Mar. 26. Urine contains no phthalein.

Mar. 26-27. Fluid feces. Phthalein excretion 64 per cent.

### URETHANE. CHLORAL.

*Dog 13-17.*—Active mongrel, male; weight 16¼ pounds.

Jan. 10. Phthalein 0.1 gm. intravenously.

Jan. 12. Phthalein excretion 61 per cent.

Jan. 20, 12 M. Dog given urethane 7 gm. by stomach tube. 1 P. M. Dog is drowsy and unable to walk. 3 P. M. Dog in stupor; slow respiration, but eyes open. 5 P. M. Urethane 2 gm. by stomach tube. Phthalein 0.1 gm. intravenously.

Jan. 21. Urine contains a definite amount of phthalein. Dog made good recovery and is normal.

Jan. 22. Abundant feces. Phthalein excretion 40 per cent.

Mar. 18, 9.30 A. M. Dog is well; weight 15¼ pounds. Chloral 5 gm. by stomach tube. 10.30 A. M. Deep anesthesia with slow respiration. 4 P. M. Deep anesthesia continues. Dog is quite cool and put on heat pad. Phthalein 0.1 gm. intravenously.

Mar. 19. Dog quite recovered. Urine contains 0.5 per cent. phthalein.

Mar. 20. Abundant fluid feces. Phthalein excretion 32 per cent.

The two preceding experiments indicate that urethane and chloral belong in the same group with alcohol. Their effect upon the liver function, with the excretion of phenoltetrachlorophthalein as an indicator, is identical.

### CHLORAL. PREGNANCY.

*Dog 13-19.*—Strong bulldog, female; weight 30 pounds.

Dec. 6. Phthalein 0.2 gm. intravenously.

Dec. 7. Fluid feces. Phthalein excretion 63 per cent.

Mar. 25, 10.30 A. M. Dog given 4 gm. of chloral by stomach tube. 11.30 A. M. Dog is drowsy and reacts slowly to stimuli. 12.30 P. M. Condition the same. Phthalein 0.2 gm. intravenously. 3 P. M. Dog recovered from effects of drug.

Mar. 27. Abundant feces. Phthalein excretion 67 per cent.

June 10. Dog is in last week of pregnancy. Weight 37 pounds. Phthalein 0.2 gm. intravenously.

June 11. Abundant feces. Phthalein excretion 61 per cent.

This experiment (dog 13-19) shows that a small dose of chloral does not depress liver function and gives some evidence that it may actually stimulate the liver excretion. This is in harmony with

other observations given above and in other papers to the effect that a small dose of a drug may stimulate the liver, whereas a larger dose may injure the liver and depress its functional activity. It is hard to show this point clearly in a normal dog, as the normal output is very close to the maximum output under any condition, and to the amount recovered in the feces after feeding the drug.

## CHLOROFORM BY STOMACH.

*Dog 13-36.*—Young bulldog, female; weight 21 pounds.

Jan. 19. Phthalein 0.15 gm. intravenously.

Jan. 20. Abundant feces. Phthalein excretion 56 per cent.

Jan. 23, 12 M. Chloroform 15 c.c. by stomach tube. 1 P. M. Dog vomited some of the chloroform. 4.30 P. M. Dog given 10 c.c. of chloroform by stomach. No vomiting. 5.30 P. M. Dog curled up quietly and shows no anesthesia.

Jan. 24. Dog quiet and not hungry. Phthalein 0.2 gm. intravenously.

Jan. 26-27. Some delay in purgation. Phthalein excretion 19 per cent.

This experiment (dog 13-36) shows the familiar effect of chloroform upon the curve of phthalein excretion by the liver. There is good evidence of injury done to the liver and interference with its capacity to excrete phenoltetrachlorphthalein. It is possible that the delay in purgation was responsible for a part of the drop in phthalein excretion, but it cannot explain this great drop in excretion which surely was dependent on actual liver injury.

## SUMMARY.

It has been established that specific liver poisons (chloroform, phosphorus) which cause histological changes in the liver cells, decrease the liver excretion of phenoltetrachlorphthalein.

Also vascular disturbances (Eck fistula, passive congestion) with or without histological evidence may cause a fall in the output of phthalein through the liver. Sufficient evidence has been brought forward to show that the phenoltetrachlorphthalein excretion is a valuable index concerning the functional capacity of the liver.

Ether anesthesia for a period of two hours usually causes a depression in the phthalein curve during the twenty-four hours following the anesthesia.

Paraldehyde in doses sufficient to give anesthesia and stupor for a few hours will give a definite fall in phthalein excretion.

Chloral and urethane usually cause a decrease in phthalein output when given in considerable amounts.

Alcohol causes a drop in the phthalein curve when given in large doses sufficient to cause stupor for a few hours. The drop in phenoltetrachlorophthalein excretion is demonstrated in the twenty-four hours following administration of the drug. A drop in the phthalein curve to two-thirds or one-half of normal indicates a definite liver injury and temporary impairment of function.



## NOTES ON THE CULTIVATION OF *TREPONEMA* *PALLIDUM*.\*

BY HANS ZINSSER, M.D., J. G. HOPKINS, M.D., AND RUTH GILBERT.

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### PLATE 21.

During the past fifteen months we have carried out a large number of cultivations of *Treponema pallidum* for the purpose of obtaining masses of spirochete material for further experimental work along a number of lines which need not be detailed in our present report. By the courtesy of Professor Fordyce, Dr. McKee, and other members of the Department of Dermatology and Syphilis of the College of Physicians and Surgeons, it has been easy for us to obtain patients with active syphilitic lesions, and we began by inoculating rabbits intratesticularly, from patients, in order to obtain material with which to work.

We have had seven successful inoculations from human beings to rabbits, but have at present in rabbits only four strains of our own, which are now respectively in the third, fourth, sixth, and eleventh rabbit generations. Although we are now engaged in attempting to perfect methods of obtaining first cultures from rabbit tissue, we have in pure culture at present but one of our own strains, strain A, with which all our preliminary cultivation work was done, and on the study of this strain, in culture, we have concentrated. This strain we have cultivated at different times from the third and fifth rabbit generations, thus excluding the possibility of its being *Treponema refringens* or some other non-pathogenic spirochete.

Since we hope to publish a separate report upon methods of first cultivation from rabbit lesions when these are perfected, we shall limit ourselves in the present communication to the discussion of

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methods of cultivation employed after the strain has once been obtained *in vitro*.

Strain A was first purified by filtration with strong suction through a Berkefeld filter, grade N. The first time we tried this, with an old, used filter candle, it happened that *Treponema pallidum* came through without accompanying bacteria. Thinking that this method would furnish a convenient modification of Noguchi's original method of culture purification, we subsequently carried out forty-four separate filtrations of mixed cultures of *Treponema pallidum* and bacteria. We hoped that *Treponema pallidum*, as in the first attempt, would come through without bacteria, either because of its greater flexibility of body or, possibly, because of the existence of a minute type of the organism, a possibility which has been discussed in the past by a number of workers. Our first experience inclined us to be very hopeful of such a result. In the entire forty-four subsequent filtrations, which were carried out with Berkefeld candles of the N, V, and W grades, as well as with a number of Chamberland F candles, and twice with Pukall filters, we never again succeeded in sucking spirochetes through the filter unless bacteria accompanied them. In these filtration experiments we employed filtration periods of from one minute to two hours and, in order to catch any possibly very young and therefore minute forms of treponemata, we employed cultures varying in age from six to forty-five days. Our results unfortunately were uniformly negative, and we believe that, incidentally, these experiments prove that we may exclude the existence of any ultramicroscopic form of the syphilitic virus, a result which confirms, for culture material, the experience of Uhlenhuth and Mulzer,<sup>1</sup> who carried out similar experiments on the filtration of material obtained from luetic rabbit testicles.

#### CULTIVATION ON FLUID MEDIA.

Our first pure cultures were grown by the well known method of Noguchi,<sup>2</sup> on heated human ascitic fluid mixed with agar in tubes, after the addition of fresh rabbit kidney, and covered with

<sup>1</sup> Uhlenhuth, P., and Mulzer, P., *Arch. a. d. k. Gsndtsamte.*, 1913, xliv, 307.

<sup>2</sup> Noguchi, H., *Jour. Exper. Med.*, 1911, xiv, 99.

sterile paraffin oil. In our early cultivations much aid was given us by the coöperation of Dr. Noguchi, who supplied us with some of his cultures for comparison. We had no difficulty in carrying along our strain on this medium as well as on gelatinized horse serum, as first used by Schereschewsky,<sup>3</sup> with and without tissue additions. In this connection it is interesting to note that we were not able to grow the culture on horse serum without tissue unless the serum had been heated and gelatinized. When the serum had been heated in this way, our strain grew both with and without the addition of tissue, and not only on the horse serum but also upon similarly prepared sheep and beef serum.

In the work that we had planned it was of the utmost importance that we should develop a fluid medium on which *Treponema pallidum* would grow readily and in large quantities. We therefore experimented first with a large number of methods modified from the published method of Noguchi, in which the plants are made in agar-serum-tissue mixtures and are allowed to grow out from this into fluid serum-salt solution-tissue mixtures. We did this most successfully finally by pouring fluid serum agar, to the height of about one inch, into the bottom of 200 cubic centimeter flasks, dropping into this sterile bits of tissue, and inoculating. When the agar had solidified, it was covered with a mixture of either salt solution and heated ascitic fluid, or slightly acid broth and ascitic fluid, up to the neck of the flask, and a few bits of sterile tissue were floated in the fluid. The flask was not sealed, but the fluid was covered, as before, with liquid paraffin oil. In such flasks large quantities of treponemata could be found free in the fluid within periods of from two to four weeks.

On further experimentation it was soon evident that it would not be necessary to continue the addition of agar to such cultures, and for the last six months we have been growing strain A directly in flasks containing serum-broth mixtures with bits of sterile fresh rabbit kidney. We have also used with success fresh rabbit spleen and fresh organs from rats and cats. The cultures from such flasks are the ones from which we are obtaining large masses of *Treponema pallidum* at present. In a subsequent paragraph we

<sup>3</sup> Schereschewsky, J., *Deutsch. med. Wchnschr.*, 1909, xxxv, 835.

shall describe our method of obtaining and of washing masses of the treponemata so cultivated. Most of our other methods in which fresh tissue is involved are merely modifications of this technique, which we think especially suitable for the production of luetin. A flask so prepared is shown in figure 1. In these flasks we have used with success not only human ascitic fluid, but also heated sheep serum, horse serum, and rabbit serum, respectively.

#### CULTIVATION WITHOUT FRESH TISSUE.

One of the chief difficulties in the cultivation of *Treponema pallidum*, as all workers who have experimented on this subject are aware, has been the occasional but unavoidable contamination of the tissue, however carefully one may remove it from the freshly killed animal.

At first we did much work in attempting to determine the factor contributed by the tissue, assuming it to depend probably upon the reducing action of the tissue enzymes. We carried out a large number of experiments in which we tried to obtain the reductase of the tissue,—if it existed,—in solution. However, extraction with water, salt solution, alcohol, etc., gave us no reducing substance apart from the tissue itself, either when the whole tissue, macerated tissue, or the juice obtained from a Buchner press was employed. We noticed, however, that while fresh tissue, under toluol, actively reduced methylene blue solutions in the course of two or three days, the same action to a slighter degree was apparent when heated tissue was so tested. In all cases the decolorizing body acting upon the methylene blue seemed adherent to the particles of tissue which settled out of the emulsions, but never seemed a property of the clear fluid used for extraction. We are inclined to think that when the heated tissue is used the decolorization of the methylene blue may be an adsorption of the dye by the tissue rather than a true reduction by abstraction of oxygen. It was noticed in similar experiments,—an observation which is not at all new, of course,—that living bacteria, which seem in contaminated cultures largely to increase the speed and amount of the growth of *Treponema pallidum*, also decolorize methylene blue, a property which in this case, as far as we can ascertain by a few simple ex-

periments, does not belong to killed cultures. It may well be that in this case the action is a true reduction.

Influenced by these experiments, but also because we thought it a procedure at least worth trying, we attempted after the tenth culture generation to grow strain A with heated tissue instead of with fresh tissue. As a result we have found that this strain would develop almost as well in the presence of the heated as in the presence of the fresh tissue. Since then we have cultivated strain A in flasks and tubes similar to those described above, together with autoclaved rabbit kidney, liver, spleen, brain, lung, and heart and skeletal muscles, in fluid media made up of mixtures of slightly acid meat infusion broth with heated sheep serum. We have no doubt that similarly good results would follow the use of ascitic fluid and horse and rabbit sera. The last named we have not employed, however, since sheep serum has been the material easiest to obtain in large quantities in a sterile condition with our present laboratory facilities.

Thinking that we might further be able to substitute pure cultures of bacteria for the tissue, we carried on experiments in this direction and have now been able to cultivate strain A in agar-sheep serum mixtures, entirely without tissue, in symbiosis with living *Staphylococcus aureus*, with *Micrococcus candidans*, and streptococcus, and in an unintentionally contaminated culture with *Bacillus faecalis alkaligenes*. This particular strain, also, grew fairly well in similar serum-agar tubes after the addition of dead staphylococci.

Encouraged by our success with the dead tissues, we then cultivated the same strain successfully upon a simple medium composed of meat juice as prepared from chopped beef in the production of meat infusion media, sterilized in the autoclave, with no other additions.

#### SUMMARY.

Successful cultures of strain A have been obtained upon the following media, with degrees of growth indicated in the following table.

Original Noguchi serum-agar-tissue .....	uniformly good growth.
Original Schereschewsky gelatinized horse serum .....	good growth.
Sheep serum, heated one half hour at 50° C., with rabbit kidney .....	good growth.

- Sheep serum, gelatinized, heated at 65° C., without rabbit kidney ..... fair growth.
- Sheep serum, gelatinized, heated at 65° C., with rabbit kidney ..... excellent growth.
- Slightly acid broth, with fresh tissue (no agar).
- With ascitic fluid ..... good growth.
- With sheep serum ..... good growth.
- With horse serum ..... good growth.
- With rabbit serum ..... good growth.
- Slightly acid broth and sheep serum with autoclaved tissue (kidney, liver, brain, lung, heart, muscle, skeletal muscle) ..... excellent growth.
- (Moderate growth of this strain occurred also with heated liver and kidney tissue in broth alone without serum.)
- Meat juice, autoclaved, without removal of clots ..... good growth.
- Symbiotic in ascitic fluid agar.
- With *Staphylococcus pyogenes aureus*, alive and dead ..... fair growth.
- With *Micrococcus candicans*, alive ..... fair growth.
- With streptococcus ..... very good growth.

We do not think that it would have been possible to cultivate strain A in these various media when first obtained *in vitro* from the rabbit, although we have no experiments to prove this, and further work alone can decide whether or not growth on the simpler media is due to a gradual development of saprophytic properties on the part of the treponemata. This, however, seems to us likely, since it is so much easier to carry along strains once obtained than it is to get them to grow at all at first.

Although the fact that we obtained this strain in culture only after it had passed through three rabbit generations excluded with reasonable certainty the possibility of our having obtained a non-pathogenic spirochete accompanying the *pallida* in the original mucous patch from which the strain was obtained, the ease with which it is now cultivated seemed to us to call for further assurance on this point, since we had unfortunately neglected to try reinoculation in rabbits with this culture at a period before the rapid attenuation, noted by Noguchi, might have occurred. We therefore sent a culture of this organism to Dr. Noguchi, who assures us that it appears, morphologically and in manner of growth, to be a typical *Treponema pallidum*. In order further to satisfy ourselves on this score before publishing our methods, we obtained, by the courtesy

of Dr. Noguchi, three of his strains, which we have labelled N. 1, N. 2, and N. 3. Experiments with these strains have shown that:

N. 1 grows well on sheep serum-broth with autoclaved kidney, liver, and brain, though not as profusely as our own strain A.

N. 2 has grown well, quite as profusely as our own, on sheep serum-broth together with autoclaved kidney, liver, brain, lung, and heart muscle, and has grown slightly on autoclaved liver and brain in broth alone without serum. It has also grown well without tissue in agar in symbiosis with living staphylococci.

N. 3, which we have recently obtained, has already grown well in sheep serum-broth with autoclaved kidney and brain, in sheep serum-broth in symbiosis with staphylococci, and in sheep serum-broth with an autoclaved clot of guinea pig blood.

Since two of these strains are of Dr. Noguchi's own isolation and the third one was sent to him and passed upon by him as a true *Treponema pallidum*, we feel safe in saying that our methods will hold good with *Treponema pallidum* generally, certainly after prolonged cultivation on artificial media. We believe that our general method of obtaining profuse growth of *Treponema pallidum* in fluid media without agar, composed of mixtures of slightly acid broth (acidity 0.2 to 0.8 per cent.) and sheep serum (or ascitic fluid, or horse or rabbit serum) containing autoclaved tissue, in long necked flasks covered with paraffin oil (figure 1), provides an excellent method of obtaining mass cultures for experimental work and for concentrated luetin preparations.

We obtain our mass cultures by cultivating for three weeks or longer in these flasks, then centrifugalizing for a short time in large tubes to remove clumps of precipitated protein, decanting, and then centrifugalizing at high speed in small tubes to throw down the organisms. Since many of the treponemata are entangled in the sediment first precipitated, a larger yield can be obtained by grinding this sediment in a mortar with salt solution and treating this material separately. The specific gravity of the treponemata appears to be rather low and there is considerable loss of material in this procedure, even after prolonged centrifugalization. Nevertheless, massive sediments of the treponemata are obtained in this way, and these can be washed in salt solution, emulsified, and used, just as are bacteria. It is noticeable also that suspensions contain-

ing very large numbers of treponemata are but slightly opaque to the naked eye.

Owing to the fact that we have had success in employing autoclaved tissue in the cultivation of these microorganisms, it has suggested itself to us that the factor in the tissue which favors the growth of the treponemata can not be of the nature of an enzyme. And we are proceeding to attempt tissue extractions with a view of obtaining apart from the tissue these essential constituents. Dr. Hopkins and Miss Gilbert are at present experimenting upon cultivations made with the aid of lipoidal tissue extracts, especially cholesterin, and preliminary experiments point favorably toward the importance of this constituent in treponema cultivation. A subsequent report will be published on this work. The possible bearing of this upon the Wassermann reaction is another of our projected problems upon which we hope shortly to be able to formulate an opinion.

We have repeatedly attempted to obtain colony growth of *Treponema pallidum* in serum agar plates with macerated tissue. There has been no question in many of these plants of the active multiplication of the treponemata; in no case have we observed true colony formation. We have, however, repeatedly observed a quasi-colony concentration of the treponemata in symbiosis with colonies of contaminating bacteria, especially *Staphylococcus aureus*. It was this observation which formed the point of departure for our attempts to cultivate *Treponema pallidum* symbiotically with bacteria.

#### CONCLUSIONS.

We consider the most important contribution reported in this paper the fact that *Treponema pallidum* can be cultivated in fluid media, without the addition of agar, together with tissues sterilized by heat. This forms an excellent method of obtaining mass cultures for luetin preparation and immunological experimentation. We may add that while the tissue varieties employed have all strongly favored the growth of the treponemata, we have noticed especially active and motile cultures when lung and suprarenal tissues were employed.

#### EXPLANATION OF PLATE 21.

FIG. 1. Flask method of growing *Treponema pallidum* in large quantities.





FIG. 1.

(Zinsser, Hopkins, and Gilbert: Cultivation of *Treponema pallidum*.)



# THE MECHANISM OF THE ABDERHALDEN REACTION.

## STUDIES ON IMMUNITY. I.\*

By J. BRONFENBRENNER, PH.D.

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The specificity of the Abderhalden test has been established by a large number of investigators who, in compliance with Abderhalden's request, worked at the test until they succeeded in obtaining the desired results. On the other hand, many other investigators have, on the basis of their experiments, questioned the specificity of the test. From the beginning Abderhalden and his pupils claimed that faults of technique were responsible for the failure to obtain satisfactory results, but the work of many investigators has shown that the reaction is no more difficult than other serological tests, and that, therefore, the explanation of the differences of the results must be looked for in other directions.

Several communications have recently appeared which attempt to find an explanation for the discrepancy of the results obtained. Flatow (1, 2, 3), Herzfeld (4), Kjaergaard (5), Plaut (6), and others reported that specific results can be obtained by proper manipulation of the material, and that one can manipulate the same material so as to obtain a positive or negative Abderhalden reaction with any serum. Flatow (3) concludes, for instance, that hardly any serum will fail to cleave placenta or lung tissue, provided a proper quantity of the substance, however well controlled, is taken.

These investigations throw a new light upon the complexity of the nature of the Abderhalden reaction, or at least they show conclusively that while the reaction may be relatively specific within certain quantitative limits, it ceases to be so outside of these limits, depending entirely on mechanical adsorption. Whether or not these experiments can sufficiently explain all the discrepancies in the results of various investigators, they show that certain quantitative manipulations may bring about phenomena closely resembling the Abderhalden reaction in a non-specific way, thus questioning the basis of the Abderhalden theory, according to which the cleavage products appear solely as a result of the specific digestion by ferments.

The experiments of de Waele (7) especially throw doubt upon the specificity of the ferments on which the Abderhalden test is supposed to depend. He suc-

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ceeded in demonstrating the presence of the substances responsible for the Abderhalden reaction a few minutes after the parenteral introduction of the foreign protein, an interval hardly sufficient for the production of new specific ferments. These results corroborate the previous findings of Heilner and Petri (8), who concluded from the rapidity with which the ferments appeared as a result of the parenteral introduction of the protein that this must be a case not of new formation of such ferments but of specific activation, "*Arteinstellung*," of preëxisting ferments. de Waele, indeed, suggests the identity of this ferment with antithrombin.

Apart from the explanation of the Abderhalden reaction on the basis of mechanical adsorption and of activation of preëxisting ferments, a number of investigators attempted to study the phenomena underlying the Abderhalden test from the standpoint of immunity. Since it was established by several workers that the complement played an important part in the reaction, the simplest explanation of the Abderhalden test would be the identification of the substances responsible for the specificity of the Abderhalden test with the antibodies. No definite proof, however, of such an identification has been offered in the numerous publications on the subject. At the same time, Abderhalden states definitely that upon the parenteral introduction of foreign protein, independently of any antibody that may be produced simultaneously, specific protective ferments are formed. In fact, the experiments of Weinland (9) and of Abderhalden and his pupils seem to show that the production of specific ferments is an even more general process than the production of antibody. For whereas antibody can be demonstrated in the cases of parenteral introduction of substances of animal or plant protein origin only, provided this protein is foreign to the species, it is claimed that the specific ferments have been demonstrated not only upon the parenteral introduction of such substances, but also upon that of proteins of homologous and even autogenous nature, provided these substances are foreign to the blood ("*blutfremd*"). Moreover, the parenteral introduction of substances like gelatin, pepton, cane sugar, or casein (10) is proved by the Abderhalden school to produce specific ferments capable of attacking said substances both *in vitro* and *in vivo*. Thus the group of substances which can play the part of antigen in the production of antibody is included in that of the substances capable of causing the production of specific ferments, but is only part of it.

Another apparently fundamental difference between the nature of antibodies and specific ferments is the mode of their action. The antibodies, although directly responsible for the specificity of the protective processes in the body through their property of anchoring the antigen, do not in themselves present any active principle, and it is to the complement that Ehrlich and his school attribute the property of acting upon the antigen. The protective ferments of Abderhalden, however, are assumed to possess the property of directly digesting the antigen, and it is on the appearance of the products of direct digestion *in vitro* that the diagnostic method of Abderhalden is based.

The experiments of Stephan (11), Hauptmann (12), Bettencourt and Menezes (13), however, seem to show that the digestive ferment in the Abderhalden test, whether specific or not in its nature, after having been inactivated by heating at 58° C. for half an hour, can be reactivated by the addition of any fresh serum

(complement). These experiments seem to be of fundamental importance, and, if definitely established, would offer a definite proof of the striking parallelism between the Abderhalden and immunity reactions in general. The part played by complement was also noticed by Kumagai (14) who succeeded in reactivating inactive serum containing invertin by the addition of the serum of normal animals, but his results were questioned by Abderhalden and Wildermuth.

The importance of the question of the part played by complement in the Abderhalden reaction is fully appreciated by Abderhalden. Referring to it he says (14):

"Nothing would be more unjustified than, on account of this parallelism, quickly to invest all the results obtained with names from the realm of immunity. For analogous phenomena need not be identical; moreover, complement, amboceptor, etc., are terms of which, in rare cases only, we have as yet a clear conception."

Thus Abderhalden, without denying the part played by complement in the test, hopes to be able to find an explanation for this without identifying his protective ferments with the antibodies of Ehrlich.

#### EXPERIMENTAL PART.

Before entering upon the specific problem under investigation I repeated some of the fundamental experiments. First I attempted to establish by experiment the specificity of the Abderhalden test in general. For this purpose two sets of experiments were undertaken, one with human sera, mainly from cases of pregnancy, in which the specific ferments of Abderhalden were supplied by patients' sera; the other with animal sera in which the specific ferments were produced experimentally previous to the actual test. My results with the Abderhalden test were strictly specific (table I), like those reported by many other workers. I adhered closely in all details to the technique described by Abderhalden (15), with a few modifications suggested in the current literature of the subject, which I adopted after many preliminary experiments.

*Technique of the Abderhalden Test. I. Serum.*—In cases of both pregnant and normal individuals the serum was taken before breakfast to avoid excess of amino acids in the blood. The blood was collected from the median vein into a large sterile Luer syringe, and immediately transferred into sterile centrifuge tubes coated on the inside with paraffin, according to Bronstein (16), and centrifuged at 4,000 revolutions per minute; the serum was separated, centrifuged a second time, and placed in the ice chest. The whole process takes one and one half to two hours.

With animals the serum was taken from the carotid artery. The blood was collected directly into a test-tube drawn out at its bottom into a narrow point which served as a cannula and was directly forced through the arterial wall.

*II. Substratum.*—Fresh human placenta within two or three hours after delivery was washed through the umbilical vein by turning the running water into the blood vessel until the water was absolutely clear (17); then the placenta was carefully dissected and all the vessels and connective tissue were removed, as directed by Abderhalden. The remaining placenta tissue was cut in very small pieces by means of a Latapie grinder and placed into a deep precipitating jar with a large amount of distilled water into which one drop of glacial acetic acid was added for each liter of water.<sup>1</sup> The tissue was energetically stirred up, allowed to settle out, and the supernatant fluid was siphoned off and replaced with a new portion of distilled water; acetic acid was added as before, and the whole was again stirred with a glass rod for two to five minutes. This washing with slightly acidified water finally removed all the remaining blood and the placenta tissue became absolutely white. Then it was transferred to boiling water and treated according to Abderhalden's method. Prepared in this way placenta often gave no ninhydrin reaction after the first boiling; if it did, however, the boiling was repeated until the boiling water contained no more substances reacting with ninhydrin. Then the placenta tissue was aseptically transferred into a sterile bottle with sufficient toluol over it to allow the stopper to touch it.

With guinea pig placenta washing through the umbilical vein was omitted on account of the small size of the organ. The whole placenta was simply washed for some time in running water, and sterilized by ultraviolet light to remove the bacteria from the surface of the organ. After this washing the placenta was dissected and treated as above.

*III. Dialyzing Thimbles.*—Thimbles of Schleicher and Schüll, No. 579 A, were used. To insure their impermeability to serum and their permeability to pepton and amino acids, they were tested according to Abderhalden's method, with the following slight modification. A serum of normal individuals was dialyzed against running sterile salt solution to insure the absence of dialyzable substances in this serum (19, 20), and 1.5 c.c. of the serum were placed into each thimble of the series, and the thimbles treated further exactly as described by Abderhalden, except that instead of using small Erlenmeyer flasks it was found preferable to use small bottles as containers for the thimbles. Their size was so selected that the height of the layer of distilled water outside and inside the thimble was nearly the same, in order to prevent the evaporation through the wall of the thimble, which might be caused by the difference of the levels in and outside of the thimble.

*IV. Test Proper.*—This was, as a rule, carried out within five to eight hours after the collection of the blood. Though experimentally no difference has been found (21), yet in compliance with Abderhalden's request only sera absolutely free of hemoglobin were used. The test was carried on in the way originally described by Abderhalden, with the exception of controls, which were made as follows: A thimble was set up with placenta covered with 5 c.c. of distilled water, and another thimble with 1.5 c.c. of the patient's serum and distilled water only, both thimbles being placed in one bottle of slightly larger size containing

<sup>1</sup> Lange (18) recommends washing in salt solution, claiming that the water leaves erythrocytic stromata in the placenta. This was, however, not found necessary, as no stromata could be found in the placenta upon careful examination by many authors.

30 c.c. of sterile distilled water (22). This control was found better than separate placenta and serum controls as described by Abderhalden, because it showed whether the combined dialysates of serum and placenta gave the ninhydrin test.

The results obtained with this technique in the preliminary experiments mentioned above are given in table I.

TABLE I.  
*Specificity of the Abderhalden Reaction.*

	Boiled proteins used as substrata.					
	Human placenta.	Human liver.	Guinea pig placenta.	Egg-white.	Beef serum.	No substratum.
Human pregnant serum . . . . .	+	—	+	—	—	—
Human male serum . . . . .	—	—	—	—	—	—
Guinea pig pregnant serum . . . . .	+	—	+	—	—	—
Guinea pig male serum . . . . .	—	—	—	—	—	—
Rabbit 75, female . . . . .	+	—	+	+	—	—
Rabbit 76, male . . . . .	—	—	—	+	—	—
Rabbit 77, male . . . . .	—	—	—	+	—	—
Rabbit 80, male . . . . .	—	—	—	—	+	—
Rabbit 81, male . . . . .	—	—	—	—	+	—
Rabbit 83, female . . . . .	—	—	—	—	+	—
No serum . . . . .	—	—	—	—	—	—

Tests for sterility were made from the contents of each thimble at the time when the dialysate was tested.

The rabbits from which the serum was taken for the experiment (table I) were immunized by repeated injections of egg-white diluted to 30 per cent. with salt solution by combining intravenous with intraperitoneal inoculations, so that on each of the dates stated below each rabbit received 5 c.c. of 30 per cent. egg-white intraperitoneally, and ten minutes later 2 c.c. intravenously.

#### INOCULATION WITH EGG-WHITE.

Rabbit 75 injected on Feb. 22, 26, Mar. 2, 6, 10, 14, 18. Bled Mar. 27.

Rabbit 76 injected on Feb. 26, Mar. 2, 6, 10, 14, 18, 22. Bled Mar. 31.

Rabbit 77 injected on Mar. 2, 6, 10, 14, 18, 22, 26. Bled Apr. 4.

Rabbit 78 injected on Mar. 6, 10, 14, 18, 22, 26, 30. Bled Apr. 8.

Rabbit 79 injected on Mar. 10, 14, 18, 22, 26, 30, Apr. 3. Bled Apr. 12.

Nine days after the last inoculation each animal was partially bled from the carotid artery, after having been kept without food for six hours. The serum of each animal was separated into two portions, one of which was used for determining the precipitating power of the serum, as a control showing that the immunization was successful; the other was used for the Abderhalden test as described above.<sup>2</sup>

<sup>2</sup> It was found necessary, on account of the large amount of amino acids in the blood of rabbits, to dialyze the blood against running salt solution before the actual test, according to Schlimpert and Issel (19), and Abderhalden and Wildermuth (20).

In a similar manner another series of rabbits was immunized against beef serum, with 3 c.c. of serum for each intraperitoneal, and 1.5 c.c. for each intravenous inoculation.

INOCULATION WITH BEEF SERUM.

Rabbit 80 injected on Feb. 24, 28, Mar. 4, 8, 12, 16, 20.	Bled Mar. 29.
Rabbit 81 injected on Feb. 28, Mar. 4, 8, 12, 16, 20, 24.	Bled Apr. 2.
Rabbit 82 injected on Mar. 4, 8, 12, 16, 20, 24, 28.	Bled Apr. 6.
Rabbit 83 injected on Mar. 8, 12, 16, 20, 24, 28, Apr. 1.	Bled Apr. 10.
Rabbit 84 injected on Mar. 12, 16, 20, 24, 28, Apr. 1, 5.	Bled Apr. 14.

Nine days after the last injection each rabbit was bled and the serum examined exactly like that of the previous series, beef serum being used instead of egg-white in the precipitation as well as in the Abderhalden test.

The sera of the rabbits of each group gave a specific Abderhalden reaction. The results of the precipitation test showed at the same time that the sera of these rabbits contained a high concentration of antibodies. It would seem, therefore, according to Abderhalden, that parallel with the production of antibody these rabbits developed specific ferments capable of digesting *in vitro* coagulated egg-white and beef serum, respectively. For as neither the serum nor the substratum, when placed in separate thimbles, contained any traces of dialyzable substances (table I), the appearance of these substances was made possible, apparently, only as a result of the cleavage of one or both of the proteins concerned, occurring as a consequence of their interaction. The fact, however, that, on the one hand, the combination of pregnant or immune sera with their corresponding substrata caused the appearance of dialyzable substances reacting with ninhydrin, while, on the other hand, the combination of the same sera with the same substrata, when attempted irrespective of specificity, was not followed by the appearance of these substances in the dialysate (table I), seems to indicate that the cleavage of protein in the Abderhalden reaction is induced by some specific mechanism. This mechanism, according to Abderhalden, is the digestion of the substratum by the specific ferments of the serum.

In order to see if immune sera in addition to the antibody actually contain such specific ferments capable of directly digesting the substratum, the following experiments were undertaken.

Mar. 31. One rabbit of the series immunized against egg-white was bled and its serum separated and tested for the ferments it contained by dipping a small



capillary tube (Mett) filled with coagulated egg-white directly into the serum; another portion of the same serum was placed in a dialyzing thimble together with about 0.5 gm. of coagulated egg-white.

After sixteen hours' incubation in the thermostat, the Mett tube was examined and it was found that no visible digestion of egg-white had taken place, whereas the Abderhalden test made with the same serum and coagulated egg-white as substratum gave a positive reaction.

Apr. 2. Rabbit 81 from the series immunized against beef serum was bled and the serum examined directly for proteolytic ferments by the following method. A regular beef serum medium was prepared, placed in small Petri dishes, and solidified in the inspissator (Loeffler), and several drops of the serum of immunized rabbits were placed on the surface of the coagulated beef serum.

If the serum of the rabbit had contained specific proteolytic ferments, it would have digested the beef serum, and at the end of digestion a small hollow should have been found under each drop. At the end of the experiment, however, it was found that the serum of the immunized rabbit left no signs of digestion of the beef serum as tested by the direct method, whereas again the Abderhalden test made with the same serum and coagulated beef serum as substratum gave a positive reaction.<sup>3</sup>

Thus the experiments suggest that it is impossible, at least by the method used, to demonstrate any direct digestion of substratum by the ferments of the serum. Nevertheless, the same sera, as mentioned above, gave a positive Abderhalden test, or, in other words, the union of each of these sera and their corresponding substrata resulted in the appearance of dialyzable cleavage products reacting with ninhydrin. If, as suggested by these experiments, the ferments of the serum did not digest the substratum, the dialyzable substance in the above experiments must have originated from the

<sup>3</sup> This experiment, as well as the one immediately preceding, of Mar. 31, in which the digestion of the substratum by the specific ferments of the serum is tested directly, is, of course, not considered adequate to decide the question definitely, since the method used is quite crude. However, it was thought advisable to perform this experiment mainly because of the fact that lately Abderhalden (23) considers that he has definitely established the fact of such direct digestion of the substratum by the use of a very similar method. Namely, he used for the test a coagulated placenta tissue, which was previously stained with carmin, and the diffusion of the stain during the test he took as a definite proof of the actual digestion of the coagulated placenta tissue by the ferments of the serum.

serum itself, as the only other source of protein cleavage present. That the substratum is not digested in the Abderhalden test and that the appearance of ninhydrin-reacting substances in the dialysate is due to the autodigestion of the serum is more conclusively shown in the following experiment.

3 c.c. of pregnant serum were placed in a centrifuge tube together with 1 gm. of boiled placenta protein, and a sufficient amount of distilled water was added to cover the placenta. The contents of the tube were covered with a thin layer of toluol, stoppered with a cork, and put into the ice box (table II, A). Parallel with this, 1.5 c.c. of the same serum with placenta were placed in a dialyzing thimble and suspended in a bottle with distilled water, as for a regular Abderhalden test, with the only difference that instead of placing it in the thermostat, it was put into the ice box (table II, B). As a control, an exact duplicate of these was put up, with male instead of pregnant serum (table II, C and D). In addition to these another control was made by placing 5 c.c. of 1 per cent. silk pepton in a dialyzing thimble (table II, E). At the end of sixteen hours the results tabulated below were obtained.

TABLE II.  
*The Abderhalden Reaction Is Arrested at 0° C.*

Pregnant human serum.		Normal male serum.		Control.
3 c.c. serum, 1 gm. placenta.	1.5 c.c. serum, 0.5 gm. placenta.	3 c.c. serum, 1 gm. placenta.	1.5 c.c. serum, 0.5 gm. placenta.	5 c.c. 1% silk pepton.
In glass tube at 0°C. for 16 hrs.	In thimble at 0°C. for 16 hrs.	In glass tube at 0°C. for 16 hrs.	In thimble at 0°C. for 16 hrs.	In thimble at 0°C. for 16 hrs.
Contents centri- fuged and ser- um separated and divided in two parts (table III).	Ninhydrin test —	Contents centri- fuged and serum separated and divided in two parts (table III).	Ninhydrin test —	Ninhydrin test +
A	B	C	D	E

While the dialysate from the pepton gave a positive ninhydrin reaction (table II, E), showing that dialysis was not arrested at 0° C., the Abderhalden test with both pregnant and normal sera gave negative results (table II, B and D), which in view of the findings with the silk pepton meant that no dialyzable substances were formed in the thimbles with pregnant as well as with male serum at 0° C.

Having thus ascertained that the digestion did not occur on ice, both centrifuge tubes containing placenta with male and pregnant

serum respectively (table II, A and C) were removed and the contents of the tubes centrifuged at high speed.<sup>4</sup> After ten minutes' centrifugation the serum was separated from the placenta in each tube and equally distributed into two thimbles each (table III, A' and A'', C' and C''). These were placed in bottles containing distilled water, the fluid inside and outside of the thimbles was covered with toluol, and one of each set of the bottles (table III, A' and C') placed in the ice box, the other (table III, A'' and C'') in the incubator. At the same time the thimbles containing a regular Abderhalden test were transferred to the thermostat<sup>5</sup> (table III, B and D). After sixteen hours' incubation at 37° C. a ninhydrin reaction was made with each dialysate, and the results obtained were those tabulated below.

TABLE III.

*The Appearance of Dialyzable Substances in Pregnant Serum after the Removal of Placenta.*

Serum A of table II.		Thimble B of table II.	Serum C of table II.		Thimble D of table II.
1.5 c.c. in thimble on ice for 16 hrs.	1.5 c.c. in thimble at 37° C. for 16 hrs.	Transferred to 37° C. for 16 hrs. in fresh distilled water.	1.5 c.c. in thimble on ice for 16 hrs.	1.5 c.c. in thimble at 37° C. for 16 hrs.	Transferred to 37° C. for 16 hrs. in fresh distilled water.
Ninhydrin test —	Ninhydrin test +	Ninhydrin test +	Ninhydrin test —	Ninhydrin test —	Ninhydrin test —
A'	A''	B	C'	C''	D

Thus it seems that by allowing the pregnant serum to combine with placenta at a low temperature (table II, A and B) it was possible to resolve the Abderhalden test into two phases, indicating that it is not a simple but a composite reaction. The experiment above shows that dialyzable substances appear only in the second phase of the reaction, after the placenta has been removed (table III, A''). The fact, moreover, that their appearance followed the combination of pregnant serum and placenta only after such a

<sup>4</sup> In order to retain the low temperature the tubes were placed in large centrifuge cups and packed around with ice.

<sup>5</sup> Before transference to the thermostat the liquid outside of the thimbles, partly used for the ninhydrin test, was brought up to its original volume, 20 c.c., with distilled water.

serum, separated from placenta, was incubated at 37° C., and that they did not appear at 0° C. (table III, A') suggests that the dialyzable substances during the second phase result from the autodigestion of the serum and not from the digestion of the placenta.

That autodigestion occurred only in the case of pregnant (table III, A'') and not in the case of normal serum (table III, C'') treated in exactly similar fashion could be explained in two ways. First it may be assumed that pregnant serum may possess proteolytic ferment, which normal serum does not contain; or again the difference could be explained by the assumption that both pregnant and normal serum possess the proteolytic enzyme, but that it is prevented from its action by some inhibiting elements which in pregnant serum are removed, and in normal serum are not. The experiments of Schwartz (24) seem to support this latter view. According to him every serum possesses proteolytic ferments which are prevented from their digestive action by the antitryptic properties of the lipoid fraction of the blood.

Recently the experiments of Plaut (6), Peiper (25), and others seem to strengthen further this view, as these authors actually succeeded in bringing about autodigestion in any serum by the addition of substances such as kaolin, barium sulphate, talcum, infusorial earth, and starch.

Independently of the question whether the conclusion drawn by these authors, that the Abderhalden test is therefore non-specific, depending merely on physical adsorption, is correct or not, their findings seem to be of sufficiently fundamental importance to repeat their experiments. Although I was not able to confirm all their findings, my results led me to the conclusion that at least kaolin, barium sulphate, and starch induce the autodigestion of the serum practically in every case. With other substances the results obtained were quite irregular, as shown in table IV.

The results of these experiments (table IV) corroborate the view that each serum may normally contain proteolytic enzyme; and the effect of the addition of the substances mentioned above can be readily understood if it is assumed, with Heilner and Petri (8), that some inhibiting substance normally present in the blood is removed by adsorption, thus setting free the preëxisting proteolytic

TABLE IV.

*Autodigestion of Serum Induced by the Addition of Different Substances.*

No. of cases.	Diagnosis.	Ninhydrin test.	0.04 gm. barium sulphate.	0.05 gm. kaolin.	0.02 gm. talcum.	About 0.008 gm. infusorial earth.	1 c.c. 0.5% agar.	1 c.c. 5% starch.	0.5 gm. placenta.	Serum alone.
4	Tuberculosis.....	+	2	4	1	2	1	4	1	1
		-	2	0	3	2	3	0	3	3
4	Syphilis.....	+	4	3	1	1	1	4	1	1
		-	0	1	3	3	3	0	3	3
4	Cancer.....	+	2	3	2	1	0	3	0	0
		-	2	1	2	3	4	1	4	4
5	Pregnancy.....	+	2	4	1	0	1	4	5	0
		-	3	1	4	5	4	1	0	5
5	Normal.....	+	3	4	0	1	1	5	0	0
		-	2	1	5	4	4	0	5	5
22	Total.....	+	13	18	5	5	4	20	7	2
		-	9	4	17	17	18	2	15	20

The technique followed in the experiment above was that of Plaut; namely, 1.5 c.c. of serum were placed in each thimble and the substances mentioned above were added. Special care was taken not to deposit any of the substances on the sides of thimbles. The substances used were freshly sterilized by dry heat, except placenta and starch which were sterilized in an Arnold sterilizer before use. The ninhydrin test on the dialysate was made after sixteen hours' incubation at 37° C.

enzymes which may subsequently digest some part of the serum. But while such a hypothesis would explain the results obtained with kaolin, starch, etc., still it would not explain the results shown in table III, where autodigestion of the serum seems to be induced by a specific mechanism, since the same amount of placenta sets free the ferment in the specific, but not in the normal serum. To explain this apparent specificity another hypothesis must be made; namely, that the substratum in contact with specific serum is so changed as to acquire the property of adsorbing the inhibiting substances, whereas in contact with normal serum no such property develops.

That the placenta actually undergoes these changes as a result of its contact with pregnant serum is demonstrated by the following experiment.

Placenta was allowed to remain in contact with pregnant serum in a centrifuge tube on ice for sixteen hours, as before. Then the contents of the tube were centrifuged and the placenta was separated from the serum. The placenta was now washed, suspended in salt solution, and placed in a dialyzing thimble,

TABLE V.  
*Removal of Serum Antitrypsin from Normal Serum by Sensitized Placenta.*

3 c.c. pregnant human serum with 1 gm. human placenta in a glass tube on ice for 16 hrs.			
Centrifuged.			
Serum separated and placed in thimble.	Placenta (sensitized?) washed and placed in thimble with 3 c.c. salt solution.		
On ice for 16 hrs.			
Ninhydrin test —	Ninhydrin test —		
Transferred to 37° C. for 16 hrs.			
Ninhydrin test +	Ninhydrin test —		
Same placenta (sensitized) separated in two parts.			
A	B	C	D
0.5 gm. placenta with 1.5 c.c. male serum in thimble at 37° C. for 16 hrs.	0.5 gm. placenta with 1.5 c.c. male serum in glass tube on ice for 16 hrs.	0.5 gm. fresh placenta with 1.5 c.c. male serum in thimble at 37° C. for 16 hrs.	0.5 gm. fresh placenta with 1.5 c.c. male serum in glass tube on ice for 16 hrs.
Ninhydrin test +	Centrifuged.	Ninhydrin test —	Centrifuged.
	Placenta in thimble with 3 c.c. at 37° C. salt solution for 16 hrs. for 16 hrs.		Placenta in thimble with 3 c.c. at 37° C. salt solution for 16 hrs. at 37° C. for 16 hrs.
	Ninhydrin test —	Ninhydrin test +	Ninhydrin test —

and the serum, separated from it, was placed in another thimble and returned to the ice box for sixteen hours. As table V shows, no dialyzable substances appeared in either case. If, however, both thimbles were now transferred to 37° C., the serum, as was shown before, gave off products of self-digestion, while the placenta did not show even traces of it. However, according to the hypothesis suggested above, such a placenta must have acquired the property of removing from the serum the substances inhibiting the activity of the ferment in the serum. To ascertain if this was the case, the same placenta was separated from salt solution and placed in contact with fresh male serum in a new thimble (table V, A). Whereas the male serum used in this experiment, when placed in contact with fresh placenta, gave a negative Abderhalden test (table V, C), the same serum, being placed in contact with the placenta previously treated with pregnant serum,<sup>6</sup> and from which all traces of the pregnant serum were washed away, gave a distinct ninhydrin reaction (table V, A).

Thus it seems that the placenta is changed by contact with pregnant serum, so that it acquires the property of removing from any serum the substances inhibiting the activity of its enzymes. Indeed the same portion of sensitized placenta is capable of starting the autodigestion of normal serum many times in succession, as was proved by the following experiment.

0.5 gm. of sensitized placenta was added to 1.5 c.c. of normal serum in a test-tube and placed in the ice box. After sixteen hours' contact the serum was separated from the placenta and transferred to a dialyzing thimble and placed in the thermostat. The same portion of the placenta was again placed with a new portion of normal serum in the ice box, and again after sixteen hours' contact the serum was separated and placed in the thermostat. The sera showed autodigestion, as evidenced by strong ninhydrin tests with the dialysate.

The removal of normal serum from the contact with sensitized placenta and the addition of fresh portions of normal serum to the same portion of sensitized placenta was repeated ten times, with the result that even the tenth portion of normal serum showed marked autodigestion when placed in the thermostat after contact with the portion of sensitized placenta used in the nine previous experiments, thus showing undoubtedly that the placenta once sensitized acquires the property of removing the antitryptic constituents of the serum in a non-specific way.

In the above experiment it was assumed that the changes undergone by the placenta were similar to those which take place when erythrocytes are placed in contact with hemolytic serum; namely,

<sup>6</sup> I propose to call such a placenta a "sensitized placenta," and the serum from which the specific properties are removed by placenta, "exhausted serum."

it was assumed that the placenta was sensitized. That such an assumption is justified can be proved by showing that pregnant serum actually loses its specific substances during contact with placenta at 0° C.

Pregnant serum was allowed to remain in a test-tube in contact with placenta for sixteen hours at 0° C. and subsequently was removed from the serum by centrifugalization.

TABLE VI.  
*Exhaustion of Antibody from Pregnant Serum.*

1.5 c.c. pregnant serum with 0.5 gm. placenta.	
In glass tube on ice 16 hrs.	
Centrifuged.	
Placenta (sensitized?) with 1.5 c.c. normal serum.	Serum (exhausted?) with 0.5 gm. fresh placenta.
In thimble at 37° C. for 16 hrs.	In glass tube on ice for 16 hrs.
Ninhydrin test +	Centrifuged.
	Placenta (sensitized?) with 1.5 c.c. normal serum.
	In thimble at 37° C. for 16 hrs.
	Ninhydrin test —

After separation from the placenta, the serum was placed in the ice box with fresh placenta (table VI). The fact that such a serum was now no longer able to sensitize this fresh placenta shows that the specific constituents of the pregnant serum (antibody?) had been removed from it.

The preceding experiments have proved conclusively that the placenta can unite with the specific substances of the pregnant serum (antibody?), thus becoming sensitized, and that such a sensitized placenta can induce autodigestion of normal serum. As to the mechanism of this process, it appears that sensitized placenta acquires the property of removing or in some other way inactivating the antitrypsin normally present in the serum, thus setting free its



proteolytic ferments. That such is the case can be proved by returning the serum antitrypsin to the serum previously exhausted of its antitrypsin and thus stopping the autodigestion. This can be accomplished by the addition of excess of normal serum (table VII).

TABLE VII.

*Inhibition of Autodigestion of Serum by the Addition of Serum Antitrypsin.*

1.5 c.c. pregnant serum, 0.5 gm. placenta.	1.5 c.c. pregnant serum, 0.5 gm. placenta.	1.5 c.c. pregnant serum, 0.5 gm. placenta.	1.5 c.c. pregnant serum, 0.5 gm. placenta.	1.5 c.c. pregnant serum alone.	0.5 gm. placenta alone.	5 c.c. serum albumen <sup>7</sup> alone in salt solution.	5 c.c. serum globulin <sup>7</sup> alone in salt solution.
Placed on ice for 16 hrs. and centrifuged at end of this time.							
Supernatant fluid transferred to a thimble and 5 c.c. of each of the indicated substances added.							
<sup>7</sup> Sodium chloride.	Normal serum.	Serum albumen.	Serum globulin.	Sodium chloride.	Sodium chloride.	Sodium chloride.	Sodium chloride.
After 16 hrs.' incubation at 37° C., the dialysates were examined for ninhydrin test.							
+	-	-	+	-	-	-	-

The work of Schwartz (24) suggests that the lipid fraction is responsible for the antitryptic fraction of normal serum. The study of this question has been undertaken in our laboratory and the results will be reported later. It seems, however, certain that the addition of serum albumen stops the autodigestion of the serum in the same measure as does the addition of the whole serum, while the globulin fraction of the serum seems to promote the autodigestion or at least to increase the amount of digested material in the dialysate.

#### DISCUSSION.

In view of the experiments reported above the fundamental points of the Abderhalden theory seem not to be substantiated. Experimental evidence speaks against the existence of specific ferments, since even the sera of highly immunized animals failed to

<sup>7</sup> Both serum albumen and serum globulin were represented by the isotonic solutions of these substances. The amount of albumen and globulin in 5 c.c. of the solution is equal to their respective quantities in 5 c.c. of blood serum.

digest directly the protein used for their immunization, and shows that the dialyzable substances appearing during the test do not originate from the substratum. It was found, on the contrary, that the ferments responsible for the cleavage of protein during the reaction are not specific; that they are present in every fresh male serum as well as in female; that the protein attacked by these ferments is not that of the substratum, but that of the serum of the patient, the Abderhalden reaction thus recording the result of the autodigestion of the patient's own serum.

As to the mechanism of this autodigestion of the serum, the following explanation can be evolved from the experiments. Proteolytic ferments are present in every fresh serum, but are normally inhibited by some antitryptic constituents of the blood. The removal of these sets free the normal trypsin of the blood, which in turn digests some part of the serum itself. Such removal of the anti-trypsin may be accomplished *in vitro* by two apparently independent processes: one non-specific,—a simple adsorption and filtering out of the inhibiting substance,—which would explain the results of Plaut, Peiper, Kjaergaard, Flatow, etc.; the other, an apparently specific process, in which the falling out or inactivation of the inhibiting substances appears to be the result of the change of the colloidal conditions of the media, resulting from the specific combination of the antigen of the substratum with the antibody of the patient's serum,—a reaction which is probably identical with that recorded also by the stalagmometer in the experiments of Ascoli. The serum deprived of antitrypsin undergoes autodigestion, which is evidenced in the Abderhalden test by the appearance of dialyzable substances.

The interaction between the substratum and the specific serum is in general comparable to the interaction between antigen and antibody in other immunity reactions, inasmuch as, apart from appearance of dialyzable substances, the substratum seems to undergo changes in the Abderhalden test identical with those of erythrocytes when acted upon by hemolytic amboceptor; namely, the substratum anchors the specific constituents of the immune serum, thus becoming sensitized. Such a sensitized substratum was found to be able to induce autodigestion in any fresh normal serum (com-

plement), and thus the important findings of Stephan (11) as to the part played by complement in the Abderhalden test can be easily explained. The heated serum containing only antibody (as the normal proteolytic ferments were destroyed by heat) retains the property of sensitizing the placenta. Upon the addition of guinea pig complement a sensitized placenta, by removing the serum antitrypsin from the guinea pig serum, liberates the proteolytic ferment of the guinea pig complement, which in turn may digest the globulin not only of the serum of the guinea pig, but also of that of inactivated human serum, thus giving rise to dialyzable substances.

The fact that a sensitized placenta can induce the digestion of the globulin in fresh serum (complement) by its proteolytic ferments, thus destroying what is supposed to be the mid-piece, may account for my observation that the complement is found to be inactivated whenever the Abderhalden test is positive. This is the subject of further study at present. It is thus possible that the complement fixation phenomenon is merely another expression of the same reaction which by Abderhalden is recorded through the ninhydrin test. Considered from this point of view, the Abderhalden theory does not seem to contribute anything essentially new to the explanation of the protective processes which take place in the body as a result of parenteral introduction of protein, but simply offers a new and effective indicator, by which the existence of the protective substances of Ehrlich can be recorded *in vitro*.

#### SUMMARY.

1. The Abderhalden reaction is specific.
2. The properties of serum on which it depends develop in experimental animals simultaneously with antibodies during the process of immunization.
3. It is impossible to observe by direct methods the presence of digesting ferments in the blood of immune animals.
4. The Abderhalden test may be resolved into two phases. A dialyzable substance appears in the second phase and is the result of the autodigestion of serum.
5. The autodigestion of serum in the Abderhalden test is due to the removal of antitrypsin from the serum by the sensitized substratum.

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# SERUM PROTEASES AND THE MECHANISM OF THE ABDERHALDEN REACTION.

## STUDIES ON FERMENT ACTION. XX.\*

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Since the Abderhalden method of dialysis has been available for clinical purposes, numerous reports of results have been published. Among these the more recent papers of Beumer (1), Fränkel (2), Csepai (3), Michaelis and von Langermarck (4), Bisgaard and Korsbjerg (5), Mosbacher and Port (6), Lange (7), von Domarus and Barsieck (8), and others, have tended to discredit the specificity of the reaction and so reflect upon its usefulness as a clinical method. The conflicting results have cast considerable doubt upon the mechanism of the reaction as first advanced by Abderhalden. It seems unfortunate that in the enthusiasm of the search for specific ferments, the proteases which might normally be present in serum and which had previously received some attention, have been neglected. We are inclined to believe that in the study of these non-specific proteases considerable information might become available which would aid in the elucidation of the points at issue in the Abderhalden reaction.

Hedin (9) first demonstrated that the globulin fraction of normal ox serum contained a weak proteolytic ferment. Later Delezenne and Pozerski (10) showed that when serum was incubated under chloroform the serum became actively proteolytic. Their experiments were not extended and received little attention. Opie (11) later demonstrated serum proteases by rendering the serum slightly acid (0.2 per cent. acetic). Delezenne and Pozerski offered no explanation for their results with chloroform, and we have only recently shown that protease action developed under these conditions is due to the fact that the serum antitrypsin is soluble in chloroform, and when so removed the serum proteases can become active (12). The method of Opie is based upon a similar phenomenon, for he found that the serum antitrypsin is inactivated in a slightly acid medium. In this manner the acid acting proteases only can become active. The inactivation of the antiferment is probably due to changes in the state of dispersion of the unsaturated lipoids induced by the acidity.

## SERUM PROTEASE.

In our study of serum protease we have noted that the serum ferment of the common laboratory animals, guinea pigs and rab-

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bits, is not specific; for when freed from antitrypsin these sera are not only autolytic but digest casein, edestin, and similar substrata. Serum from the dog, cat, and ox show considerable variation. Thus in normal dog serum proteases could be demonstrated only occasionally, but appeared regularly under pathological conditions (distemper, pneumonia, and inanition). The serum protease of these animals acts both in a slightly acid as well as in an alkaline medium; its action, like that of trypsin and leucoprotease, is inhibited by diluted solution of the unsaturated soaps, and the thermal death point lies near  $70^{\circ}\text{C}$ . when heated for thirty minutes, although lower temperatures ( $56^{\circ}\text{C}$ . for thirty minutes) cause marked impairment of the proteolytic power. The relative thermostability as compared to serum complement is of considerable importance both from a theoretical as well as a practical standpoint. The non-identity of serum complement and serum proteases should be emphasized, for throughout the literature these ferments are constantly confused. The simplest demonstration of the difference is made by the action of chloroform upon guinea pig serum. When this serum is thoroughly mixed with chloroform and incubated, the complement and esterase are rapidly destroyed, whereas the serum protease becomes active under these conditions, as first noted by Delezenne and Pozerski. Complement action is constantly referred to as proteolytic, and it is assumed that the temperature which inactivates complement also inactivates serum protease. Thus Lange (13) in his excellent study of the Abderhalden reaction is unable to account for the fact that frequently he noted that inactivated serum gave a positive reaction, although somewhat weaker than the active serum. Assuming that all proteolytic activity had been destroyed with the inactivation of the complement, he could not explain these reactions, although it becomes evident that such weak reactions will be observed frequently when tested for, inasmuch as at  $60^{\circ}\text{C}$ . the protease is impaired and not completely destroyed. Williams and Pearce (14) have recorded similar observations. It is shown with guinea pig serum incubated with placenta for twenty-four hours as follows:

	Dialysate. Ninhydrin reaction.
1. Normal guinea pig serum plus placenta .....	+++
2. Guinea pig serum (inactivated at 56° C. for 30 min.) plus placenta	+
3. Guinea pig serum (inactivated at 70° C. for 30 min.) plus placenta	o
4. Guinea pig serum (inactivated at 56° C.) .....	o

A similar result was obtained when the serum was incubated with starch or with chloroform.

We have previously indicated (15) that the complemental activity of the serum is probably related to the esterases and, therefore, bears no direct relation to the Abderhalden reaction. The effort has been made to reactivate heated specific sera by adding guinea pig complement, and in this several observers have claimed positive results. Inasmuch as they are in this way adding a large amount of a non-specific protease, these results can have no value.

In contrast to the serum of the smaller laboratory animals, we have found that normal human sera contain little or no protease. Flatow (16), on the other hand, contends that all sera contain protease in considerable amounts, which he has demonstrated by adding casein to serum in dialyzing membranes. He found that the ninhydrin reaction was positive with all sera and was simply increased in pregnancy. These experiments have, however, the serious objection that casein is rather easily hydrolyzed to the higher splitting products without the presence of any protease, and the serum ereptase or peptase, which is undoubtedly present in all sera, would then digest the higher splitting products to dialyzable forms. The differentiation between serum protease and the peptase or ereptase is at times difficult to draw, although it is of primary importance.

It is at any rate interesting that animals which have a constant and strong protease in their serum (guinea pigs and rabbits) should be without such ferments in the leucocytes, whereas animals in which leucocytic proteases are well developed (dogs and man) are seemingly under normal conditions without much protease in the serum.

#### ABDERHALDEN REACTION.

From a theoretical point of view there are several phases in the explanation of the Abderhalden reaction which are at variance with

various facts in immunological research as developed in the past few years. It has been found that in those conditions in which the Abderhalden reaction has been most frequently tested for,—pregnancy, carcinoma, tuberculosis, and the various nervous lesions,—there is present a marked increase in the antiferment of the serum. The increase in pregnancy (and carcinoma) is so well recognized that numerous workers have suggested the use of the antitrypsin titer in place of the Abderhalden reaction.

Thus Franz (17) in a series of 223 cases and Heinemann (18) in a series of 50 cases report better results than with the Abderhalden method. This has been taken up fully by Freund and Brahm (19), who came to the same conclusion. Under such conditions we should expect less digestion of native placenta in the serum than normally. This, as a matter of fact, is exactly the condition found. Wilhelm and Szandicz (20) have recently shown that native placental cells (not boiled) autolyzed in normal serum, but that the serum of pregnant individuals retarded this autolysis, because of the increase in antiferment. These facts are, of course, contrary to the theory of the Abderhalden reaction. Again, the idea is advanced that as a result of tissue destruction or of infection, lytic bodies or proteases are found which are capable of splitting the infecting organism (as in tuberculosis) or the cell (as in carcinoma). Now it is a well established fact (reaction of Freund and Kaminer) that in carcinoma we have actually the reverse of this process; *i. e.*, the blood of the carcinoma patient has lost the power to dissolve carcinoma cells normally possessed by the serum. Yet the Abderhalden theory is based on the diametrically opposite supposition without any experimental basis in its support.

Even if we leave aside the question of the impairment of specificity of boiled tissues, one rather striking feature stands out in reviewing the results obtained in various laboratories, in that placental tissue is digested by practically all pathological sera irrespective of pregnancy, whereas the cross-digestion of caseous material and of carcinoma tissue is much less constant. It has been noted that normal lung tissue next to placental tissue is most frequently digested. The reaction is obtained only when a formed substrate is used, and seems to depend on the mechanical state of division of that substrate; or if a fluid substrate is used, it has been found that the reaction is positive only when a precipitin reaction occurs simultaneously. Thus there would seem to be some dependence of the reaction upon physical factors.

This is indicated in the work of Plaut (21) who found that with guinea pig serum the Abderhalden reaction was regularly obtained not only with placental tissue but also with inert substances such as kaolin, *Kieselguhr*, barium sulphate,



etc. This work, of course, showed that digested products might be derived from the serum and not from the added substrate. We have previously demonstrated such digestion if serum is extracted with lipoidal solvents (22). In a manner similar to that of Plaut, Peiper (23) and Friedemann and Schönfeld (24) regularly obtained a positive Abderhalden reaction when starch was added to the serum. Probably the most suggestive work is that of de Waele (25) who found that any agent which would cause an alteration of the physical state of the serum globulins would cause a most intensive Abderhalden reaction, and concluded that the reaction depended upon a globulinolysis, having an origin in processes possibly analogous to the precipitin reaction. In this it is interesting to note that Eggstein (26) found that the mere dilution of the serum in the dialyzing membrane with a large amount of distilled water would frequently give a positive reaction without the addition of any substrate, the result being probably due to a precipitation of the globulins. He also found that an acid reaction interfered with the reaction, whereas in an alkaline medium the digestion seemed increased. This has been noted by Goormaghtigh and Deheegher. In view of these results the view would seem warranted that dialyzable products responsible for the Abderhalden reaction might originate from the serum and that the phenomenon depended upon adsorption processes, the substrate added—placental tissue, caseous material, bacteria, etc.—acting as adsorbing media and not as substrata. On the basis of our work with the serum antiferment and on the serum lysis of bacteria, we have previously (15) suggested this explanation of the Abderhalden reaction.

We have recently demonstrated that the serum antiferment is a readily adsorbable substance, being adsorbed by bacteria, kaolin, starch, agar, etc., and that following such adsorption the serum proteases normally present may split the serum proteins to toxic products, as in anaphylatoxin formation (27). It would then seem logical to investigate whether similar processes underlie the Abderhalden reaction. Under such circumstances we should expect that (a) the placental tissue would not be decreased in amount during digestion, (b) the placental tissue used in the reaction would become more resistant to tryptic digestion because of adsorption of antiferment, (c) the serum in the dialyzing membrane would show some lowering of its antiferment titer, (d) the developed protease action would be non-specific, and (e) that other means of adsorption of the antiferment would reveal the presence of proteases.

#### EXPERIMENTAL.

##### LACK OF PLACENTAL DIGESTION BY SERUM.

In order to demonstrate that the placental tissue is not digested we have carried out the following experiment.

Placental tissue was dried according to the method of Lindig (28). 70 mg. were made up into an emulsion, boiled, and carefully divided into ten centrifuge tubes. 2 c.c. of pregnant serum were added to five tubes, and an equivalent amount of salt solution to the five control tubes. After digesting for twenty-four hours the placental tissue was centrifuged from the supernatant fluid, and after two washings the total nitrogen of the placental rest was determined. The non-coagulable nitrogen of the supernatant fluids and washings was also determined.

Total non-coagulable nitrogen per c.c. of serum..... 0.25 mg.  
 Total non-coagulable nitrogen of the salt solution from placenta..... 0.05 mg.  
 Total non-coagulable nitrogen per c.c. of serum from placenta..... 0.37 mg.  
 Total nitrogen of placental tissue from salt solution controls..... 0.58 mg.  
 Total nitrogen of placental tissue from serum tubes..... 0.74 mg.

Instead of a lessened amount of substrate, as would be demanded if the placental tissue were actually digested, there is an actual increase in the amount of nitrogen-containing material which has been derived from the serum, while there is at the same time an increase in the total non-coagulable nitrogen of the supernatant serum, the split products being formed from the serum proteins.

#### RESISTANCE OF PLACENTAL TISSUE.

That the placental tissue becomes more resistant to enzyme action following the dialysis is shown in the following experiment.

Dried placental substrate was suspended in salt solution so that 1 c.c. contained 2 mg. of nitrogen. A similar preparation was made from placental tissue after it had been subjected to the action of serum and thoroughly washed free from the serum. The non-coagulable nitrogen in each suspension amounted to 0.03 mg. per c.c. Both suspensions were made alkaline with sodium carbonate, and 0.1 c.c. of trypsin solution was added, an amount sufficient to digest 2 c.c. of 1 per cent. casein solution in one hour. The amount of digestion noted was as follows.

	Digestion in per cent. after	
	1 hr.	4 hrs.
Untreated placenta.....	2.5%	18%
Serum placenta.....	0.6%	10%

The serum-treated placenta had become almost twice as resistant as the normal placenta.

#### ADSORPTION OF ANTIFERMENT.

Protease action in the serum must take place under conditions

of antiferment deficiency, for if the ferment action were not over-balanced by an antiferment the organism would die immediately from intoxication from the protein split products. We believe that the antiferment deficiency need not be expressed by a lowering of the titer of the whole serum unless the adsorption has been very extensive. Indeed, it seems probable that the protease action can take place in what might be termed local areas of antiferment deficiency, such as must occur at the point of contact of the serum and adsorbing substance. A complete absence of antiferment is, therefore, not essential for protease action provided some adsorbing surface is present on which the relative balance of ferment-antiferment may be altered. The titer of the serum is, however, lowered as a whole during the dialysis, as is shown in the following experiment with pregnant serum which was incubated for thirty-six hours and which gave a +++ Abderhalden reaction.

Serum dilution.	Inhibition of serum.	
	Before dialysis.	After dialysis.
0.10 c.c.	90%	91%
0.075 c.c.	91%	90%
0.05 c.c.	91%	91%
0.025 c.c.	91%	33%
0.01 c.c.	24%	10%

It will be observed that an excess of serum is able to cause almost complete inhibition of the trypsin unit, the lowering of the titer being evident in the greater dilutions.

It is probable that the dialyzing membrane itself acts to a certain extent as an adsorbing membrane for the antiferment, so that frequently a serum, which in itself does not contain sufficient dialyzable substances to give a positive reaction, as evidenced by the negative control of inactive serum, and also of inactive serum plus placenta, will give a positive reaction. When placed in glass such a serum will not undergo autolysis. Paul Lindig (28), among others, has noted this phenomenon, and concludes that the dialyzable products are due to enzyme action and not to preformed dialyzable products. Such an experiment is shown in the following protocol.

	Abderhalden reaction.	Antiferment titer after dialysis, for 0.025 c.c.	Total nitrogen in dialysate.
Pregnant serum.....	+	45%	0.118 mg.
Pregnant serum inactive.....	0		0.13 mg.
Pregnant serum plus placenta.....	+++	33%	0.25 mg.
Pregnant serum inactive plus placenta...	0		0.17 mg.
Placenta.....	0		0.07 mg.
Undialyzed serum.....		90%	Total non-coagulable nitrogen in serum 0.12 mg.

There has been a considerable reduction of the titer of the serum in the dialyzing membrane even without the addition of substrate. It will be recalled that this adsorption of the antiferment by dialyzing membranes led to an error on the part of Rosenthal, who sought to show that the antiferment of the blood consisted of the dialyzable protein split products, and concluded that the reduction of antiferment following dialysis was due to diffusion of the split products.

#### NON-SPECIFICITY.

The fact that pregnant and various pathological sera will, when placed in the dialyzing membrane with inorganic or organic agents, give a positive Abderhalden reaction has already been demonstrated, the most recent series being that of Freund and Brahm (19), who noted that in fifty-eight cases they obtained positive results in forty cases. Similar results have recently been described by Bronfenbrenner (29) with the use of chloroform. He concludes that the serum itself is the source of the dialyzable products, that the placental tissue is not digested, that substances which removed the antiferment,—chloroform, kaolin, starch,—cause a positive reaction, while the addition of antiferment causes the inhibition of the reaction. Bronfenbrenner has noted that the intensity of the Abderhalden reaction is inversely related to the complement activity, thus offering additional evidence of the fact which we have endeavored to emphasize; namely, the non-identity of the serum complement and serum protease. Bronfenbrenner does not, however, draw the same conclusion from this observation.

In our own experiments we have obtained positive results with pregnant, tuberculous, and pneumonic sera, whether chloroform, agar, starch, or placenta were used as adsorbing media. With agar, starch, and chloroform the effects were less uniform when these substances were placed directly into the dialyzing membrane, probably because they interfere more or less with the rapidity of diffusion. When, however, these substances were permitted to act

upon the serum at an incubator temperature for a period of time, and the serum was then placed in the thimbles, a positive result was invariably obtained. The interference of chloroform and of the colloids with the dialysis is shown in the following experiment in which 1.5 c.c. of Seiden peptone were permitted to dialyze in one membrane, and chloroform and agar were added to an equal amount of peptone in other membranes.

	Abderhalden reaction.	Per cent. of nitrogen dialyzed.
Peptone.....	+++	82%
Chloroform and peptone.....	++	58%
Agar and peptone.....	+	52%

This effect of the colloids must be considered when experiments are made by adding such substances directly to the thimbles. We have found it advisable to permit the antiferment adsorption to take place before the serum is dialyzed, the adsorbing substances being centrifuged from the serum as much as possible before the serum is placed in the thimbles. Even a short period (three hours at 37° C.) of the adsorbing action may in some cases be sufficient to give a positive reaction, as is shown in the following experiment with pneumonia serum.

	Abderhalden reaction.	Total nitrogen in dialysate.
1. Serum.....	0	0.34 mg.
2. Inactive serum.....	0	0.3 mg.
3. Serum and placenta.....	++++	0.56 mg.
4. Inactive serum and placenta.....	++	0.41 mg.
5. Serum and chloroform.....	+	0.33 mg.
6. Serum and agar.....	0	0.28 mg.
7. Serum and starch.....	+	0.37 mg.
8. Serum and kaolin.....	0	0.2 mg.

1.5 c.c. of serum were used. The placental tests were permitted the usual time of twenty-four hours. Tests 5, 6, 7, and 8 were mixed with chloroform (equal volume), agar (equal volume of 0.1 per cent. solution), starch (equal volume of 10 per cent. solution), and kaolin (0.05 gm.) and incubated for three hours. They were then centrifuged and the clear sera placed in the thimbles over night.

It is probable that the negative reactions with the agar and kaolin were due to insufficient amounts of adsorbing substances. We have discussed this point in a previous paper (27). In all the sera

tested (pregnancy, pneumonia, and tuberculosis) the placenta has given a positive reaction, while caseous material has given a cross-reaction with both pregnant and pneumonia serum. An extended series of sera from various pathological cases has been reported by Falls (30), who in eighty-four pathological sera obtained positive reactions varying from weak to strong in sixty-eight, using placental tissue as a substrate. Under such circumstances the specificity of the reaction is at least highly doubtful.

In view of the experimental data presented above, together with those given in our previous papers, we are inclined to believe that the Abderhalden dialysis method and the theory underlying it, in so far as it is applicable to protease action, is without warrant of specificity, and probably depends upon purely fortuitous mechanical factors. It seems probable that in various pathological conditions proteases normally confined to the leucocytes in the human being appear in the blood where their presence can be demonstrated by a method which removes the antiferment without injuring the ferment. The proteases are not specific, the placental tissue being found most efficacious, possibly because of purely mechanical factors (surface exposure), as is indicated by the wide range of clinical conditions in which the placental substrate gives positive results.

#### CONCLUSIONS.

1. Normal serum protease is not specific; it is active in both dilute acid as well as alkaline media. It is destroyed by heating to 70° C. for thirty minutes. It is markedly impaired when heated at 56° C. for thirty minutes. It is inhibited by the unsaturated soaps and lipoids.
2. Guinea pig and rabbit serum contain relatively much protease; the leucocytes are without proteolytic ferments.
3. Normal human and dog serum contain little or no protease; the leucocytes are strongly proteolytic.
4. Serum complement and protease are not identical.
5. During various pathological conditions the non-specific protease is increased in both human and dog serum.
6. An increase in antiferment is in many instances coincident.
7. During the Abderhalden reaction the placental tissue becomes more resistant to enzyme action, because of the adsorption of the antiferment from the serum.

8. The dialyzed serum loses antiferment because of adsorption by the placental tissue or other adsorbing substances, including probably the dialyzing membrane.

9. The digestive substrate is the serum protein made available for protease action by the adsorption of the antiferment.

10. The proteases in pathological conditions investigated by us (pregnancy, tuberculosis, and pneumonia) are non-specific.

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THE INFLUENCE THAT SERUM EXERTS UPON TRY-  
PANOSOMES, WITH SPECIAL REFERENCE TO  
ITS USE FOR EXPERIMENTS IN VITRO  
WITH ATOXYL AND PARAMINO-  
PHENYLARSENOXYD.\*

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INTRODUCTION.

Although it has been known for many years that trypanosomes suspended in serum may retain their motility for days, it apparently occurred to no one to make use of this fact in experiments with trypanosomes *in vitro*. Nevertheless, a good medium for suspending trypanosomes was needed, for salt solution which has usually been employed in experiments *in vitro* is so poisonous for these organisms that it exerts on them a marked toxic influence, and for this reason limits the duration of the experiments. The use of serum for experiments *in vitro* was, however, not even suggested by Schern,<sup>1</sup> who in 1911 published a paper on the activating properties of serum and liver extracts. Schern pointed out that this property in serum could be retained for a long time.

The advantage of using serum for experiments *in vitro* with nagana (goat normal passage iii) I discovered quite by accident while working in 1910 in the George Speyer House in Frankfurt a/M. In testing the toxicity for trypanosomes of paraminophenylarsenoxyd I found that the motility of the trypanosomes was preserved better in certain weak dilutions of the toxin dissolved in blood than in the controls made by suspending the organisms in salt solution.

This surprising fact was at once investigated and a stimulating influence due to the toxin was excluded, for similar dilutions in

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<sup>1</sup> Schern, K., *Arb. a. d. k. Gsndhtsamtc.*, 1911, xxxviii, 338.



salt solution did not have this effect. The blood was examined and experiments soon showed that the effect seen was due to the serum in the blood and to the serum alone.

As I had been seeking in vain for a substitute for salt solution for experiments *in vitro*, it occurred to me that serum might be used for this purpose. I tried it and found it so satisfactory that it has been employed since then in nearly all my experiments.

Although serum was used in my experiments throughout 1910 and 1911, I did not report its advantages for experiments *in vitro* until February 21, 1912.<sup>2</sup> A few days later there appeared in Germany a paper by Rothermundt and Dale.<sup>3</sup> In this paper Rothermundt and Dale called attention to the value of suspending trypanosomes in serum. By so doing they had been able to prolong their experiments *in vitro*, and prolonging their experiments had, they said, enabled them to discover what others had overlooked; *i. e.*, that atoxyl has a direct action upon trypanosomes *in vitro*.

In my paper in 1912 no details were given. In this one I desire to publish a few protocols and add to the points made in 1912.

Serum preserves the motility of trypanosomes suspended in it much better and longer than salt solution does. In one experiment a richly infected mouse was bled to death and its blood was divided in two parts, one part being added to salt solution and the other part to serum. The trypanosomes in serum apparently lost none of their activity during the three hours they were observed. Those placed in salt solution lost in activity at once. After 25 minutes they were only about 30 per cent. as active as those in serum, and after 2 hours and 5 minutes at room temperature they were completely immobilized. This experiment was repeated many times, and except for slight differences in detail the result was always the same. In all experiments serum was superior to salt solution in preserving the motility of trypanosomes.

Various sera.—rabbit, ox, horse, goat, sheep, pig, chicken, rat, and mouse,—were tested for their motility-preserving properties, and all of them were found to possess it. In experiments with rat and mouse sera some trypanosomes were found to be 100 per cent.

<sup>2</sup> Terry, B. T., *Proc. Soc. Exper. Biol. and Med.*, 1911-12, ix, 40.

<sup>3</sup> Rothermundt, M., and Dale, J., *Ztschr. f. Immunitätsforsch., Orig.*, 1912, xii, 565.

motile even after 24 hours' contact with the serum at room temperature.

To ascertain how strong this motility-preserving property was, salt solution in varying quantity was added to the sera and the tests were repeated. These tests showed that some slight effect of the serum was seen in dilutions as great as 1 part in 16 or 1 in 32, but that dilutions of serum greater than 1 in 2 or 1 in 4 were not as good as undiluted serum if the contact at room temperature was 3 hours or longer.

Serum obtained from the slaughter house was rarely or never sterile, and infected serum soon proved unsatisfactory. To free the serum from bacteria a sterile Berkefeld filter was employed, and the filtered serum was tested. It was found to be perfectly satisfactory.

To ascertain how long filtered serum would preserve its properties, a large quantity was filtered, bottled aseptically, and preserved in the ice box for many months. In serum that had been preserved nearly a year no diminution in the motility-preserving property was seen. It became easy, therefore, to keep on hand an abundant supply of active serum.

"*Salz physiologicum*" of Merck was tested, at the suggestion of Professor Ehrlich, to see if it would be as effective as serum. It proved to be far inferior and seemed to be no better than the ordinary 0.9 per cent. pure sodium chloride solution used in the laboratory.

At the suggestion of Dr. Meltzer I experimented, after my return to America, to see if Ringer solution might not give as good results as serum. In my experiments I used two different Ringer solutions.<sup>4</sup>

<sup>4</sup>

RINGER SOLUTION, MELTZER.

Sodium chloride .....	9.00 gm.
Calcium chloride .....	0.22 gm.
Potassium chloride .....	0.30 gm.
Sodium bicarbonate .....	0.2 gm.
Magnesium chloride .....	0.095 gm.
Distilled water, made up to .....	1,000.00 c.c.

This was prepared by Dr. Auer and furnished by Dr. Githens.

RINGER SOLUTION, CARREL.

Sodium chloride (Merck, highest purity, pure, fused) .....	9.0 gm.
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In the first Ringer solution experiment a mouse infected with trypanosomes was decapitated, its blood was defibrinated, and 0.1 of a cubic centimeter was added to each of four tubes, each of which contained five cubic centimeters of fluid. The first tube contained salt solution (0.9 per cent.), the second the Meltzer Ringer solution, the third the Carrel Ringer, and the fourth ox serum. The result of the tests is shown in table I, the motility of the trypanosomes being expressed in percentages, 100 per cent. being unimpaired motility. Smaller percentages show correspondingly decreased motility.

TABLE I.

No.	After contact for	Salt solution. Motility per cent. <sup>5</sup>	Ringer, Meltzer. Motility per cent.	Ringer, Carrel. Motility per cent.	Ox serum. Motility per cent.
7.187,8	5 min.	60	60	100	100
7.187,8	30 min.	35	26	100	90
7.187,8	60 min.	10	10	60	90
7.187,8	120 min.	5	5	60	90

From table I we see that after 5 minutes no difference was evident between the Carrel Ringer solution and the serum, and that these were distinctly better than the Meltzer Ringer and the salt solution. The cattle serum in this experiment seemed, however, to be a little less efficient than usual, and after 30 minutes the Carrel Ringer appeared to have preserved the motility a trifle better than the serum.

After 60 and 90 minutes, however, the serum was undoubtedly better than the Carrel Ringer solution, and the Carrel Ringer was distinctly better than the Meltzer Ringer and the salt solution.

About 6 weeks later the experiment was repeated with fresh samples of salt solution and Ringer solution. The result is shown in table II.

Calcium chloride .....	0.25 gm.
Potassium chloride .....	0.42 gm.
Distilled water .....	1,000.00 c.c.

The salts are weighed out and dissolved in 1,000 c.c. of distilled water.

Prepared and furnished by Mr. Ebeling.

<sup>5</sup> The motility of the trypanosomes was observed under the microscope, described quickly by means of abbreviations, and these abbreviations were subsequently converted into percentages by rules which insure a given description yielding the same result every time. These rules will be given in another paper to appear soon.

TABLE II.

No.	After contact for	Salt solution. Motility per cent.	Ringer, Meltzer. Motility per cent.	Ringer, Carrel. Motility per cent.	Cattle serum. Motility per cent.
7.188	15 min.	76	40	76	100
7.188	45 min.	33	5	5	100
7.188	165 min.	2	0	0	100

In the second experiment the Carrel Ringer solution proved to be distinctly inferior to serum, almost as good as salt solution, but distinctly superior to the Meltzer Ringer.

In other experiments with Carrel Ringer solution, salt solution, and serum, the serum gave the best result and the Carrel Ringer proved slightly inferior to salt solution. Ringer solution is, therefore, apparently inferior to serum in preserving the motility of trypanosomes.

In the experiments with Ringer solution many rounded trypanosomes were observed in the fresh specimens. It seemed desirable for this reason to stain some of the parasites. Accordingly after 3 hours' contact the trypanosomes in Ringer solution and serum were spread on a slide and stained by the Giemsa method. Most of the trypanosomes in the Meltzer and Carrel Ringer solutions were found to be more or less completely disintegrated. At times flagella attached to centrosomes were seen, the rest of the organism having disappeared. Rounded forms containing nucleus, centrosome, and partially freed flagellum were also present.

In the fresh specimens and in stained specimens made from the serum suspensions none of these abnormal forms were seen. Serum appears, therefore, to preserve both the motility and the normal morphology of trypanosomes better than either of the Ringer solutions tested.

To forestall the criticism that the ox serum I was employing might have some deleterious effect that was not evident in altering the motility or morphology, I tested comparatively, for their infectiousness, trypanosomes preserved in salt solution and trypanosomes kept in serum. These experiments showed that under both conditions trypanosomes preserved 2, 4, and 6 days at room temperature were infectious for mice, but that the trypanosomes preserved in serum infected sooner and killed the animals more quickly

than those preserved in salt solution. For example, the parasites preserved in salt solution for 6 days appeared in the blood of a mouse 9 days later and killed it on the 11th day. Under similar conditions trypanosomes preserved in serum were visible in the blood of a mouse on the 4th day and killed it on the 7th day. In one instance trypanosomes preserved in serum for 8 days at room temperature were injected into a mouse and the organisms infected and killed this animal as quickly as trypanosomes which had been preserved under similar conditions in salt solution for only 6 days.

That trypanosomes were still infectious after being kept at room temperature for 8 days in cattle serum suggests the possibility of using serum suspensions for transporting virus where for one reason or another it may be inconvenient to use an infected animal. Eight days is long enough to transport virus from New York to San Francisco, or even from New York to London, Paris, or Frankfurt.

*Ice Box Temperature.*—A few experiments were made to see if the infectiousness of trypanosomes would be better preserved at ice box temperature than at room temperature. In every instance, however, room temperature seemed better than ice box temperature, but even at ice box temperature the infectiousness of the trypanosomes seemed to be preserved better in serum than in salt solution.

Before serum could be employed in my experiments *in vitro* with atoxyl and paraminophenylarscnoxyd it was necessary to determine that serum would not exercise any disturbing effect. A number of experiments were therefore carried out.

Serum was tested for its transforming power in the same way that blood had been tested. A 10 per cent. solution of atoxyl in rabbit serum was incubated at 37° C. for 3 hours, and then trypanosomes were brought in contact with various dilutions of the atoxyl dissolved in serum. The result is shown in outline.

No.	Method.		After contact for	Motility per cent.	Dilution.
5.69	Rabbit serum	A (10%, 37°, 3 hrs.)	65 min.	100	100
5.68	Rabbit blood	A (10%, 37°, 3 hrs.)	60 min.	0	3,000

From this we see that after 65 minutes' contact with serum containing one part of atoxyl in 100, the trypanosomes were uninfluenced, whereas in the control experiment in which rabbit blood was employed instead of serum, all the parasites were immobilized in 60 minutes in an atoxyl dilution of 1 part in 3,000. Serum, therefore, apparently does not transform atoxyl.

The binding power of serum for paraminophenylarsenoxyd, one of the reduction products of atoxyl, was also tested. The tests were made in a number of ways, but the results agreed in indicating that serum did not in any wise lessen the toxicity of paraminophenylarsenoxyd for trypanosomes.

The following outline will show the extreme toxicity of paraminophenylarsenoxyd when added to twice its volume of serum.

No.	Method.	After contact for	Motility per cent.	Dilution.
2.63	1 c.c. serum + 0.5 c.c. pax + 0.5 c.c. tryps.	30 min.	0	1,000,000
2.63	1 c.c. serum + 0.5 c.c. pax + 0.5 c.c. tryps.	38 min.	0	2,000,000

Pax is the abbreviation for paraminophenylarsenoxyd; tryps., for trypanosomes.

From the outline we see that in 30 minutes all the trypanosomes were immobilized in a paraminophenylarsenoxyd dilution of 1 to 1,000,000, and in 38 minutes in a dilution of 1 to 2,000,000. The toxicity shown here by paraminophenylarsenoxyd dissolved in serum and acting for 30 minutes on trypanosomes, is exactly like that which Ehrlich<sup>6</sup> reported in 1908 for the same medicament dissolved in salt solution and acting on trypanosomes for 30 minutes.

In a number of experiments trypanosomes were immobilized more quickly in serum than in corresponding dilutions of salt solution. This was apparently due to the fact that in the presence of serum trypanosomes move more rapidly than in the presence of salt solution. By increasing the metabolic processes of the trypanosomes, serum probably causes the parasites to take up the poison more rapidly and to show its effects more quickly than they do when suspended in salt solution.

<sup>6</sup> Ehrlich, P., *Verhandl. d. deutsch. dermat. Gesellsch., Xte. Kongr.*, 1908, 52-70.

In conclusion I desire to express my sincere thanks and deep obligation to Professor Ehrlich for supplying me so freely with virus, medicaments, and animals, and for the opportunities he afforded me of following up the observation I made while working on a problem he had suggested.

#### SUMMARY.

1. Serum of various animals preserves the motility of nagana trypanosomes better and longer than salt solution.
2. To act best in this way the serum should not be diluted more than 2 to 4 times. Undiluted serum is perhaps best.
3. Serum filtered through a Berkefeld filter, bottled aseptically, and kept in the ice box preserves this activating property apparently undiminished for many months.
4. Serum preserves the motility of trypanosomes better than "*Salz physiologicum*" of Merck, and better than the Ringer solutions of Meltzer and Carrel.
5. Serum preserves the normal morphology of trypanosomes better than the Ringer solutions tested.
6. The infectiousness of trypanosomes suspended in cattle serum was preserved at room temperature for at least 8 days.
7. The vitality of the trypanosomes in serum was seemingly better preserved at room temperature than at ice box temperature.
8. Serum incubated with atoxyl does not transform it into a toxic substance.
9. Serum does not bind paraminophenylarsenoxyl, for trypanosomes suspended in serum are often immobilized more quickly by paraminophenylarsenoxyl than trypanosomes suspended in salt solution.
10. Serum is suitable for suspending trypanosomes for certain experiments *in vitro*, and with proper precautions may be employed for transporting virus from laboratory to laboratory.

DIFFERENT AMOUNTS OF TRANSFORMED ATOXYL  
PRODUCED BY INCUBATING ONE PER CENT.  
AND TEN PER CENT. ATOXYL IN BLOOD.\*

By B. T. TERRY, M.D.

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INTRODUCTION.

Atoxyl in 5 per cent. solution *in vitro* has at room temperature no influence on trypanosomes for several hours, but dissolved in blood and incubated at 37° C. for 2 or 3 hours it gives rise to a substance (transformed atoxyl, or trypanotoxyl) that is quite toxic for these parasites.

This toxic substance has probably not been isolated in purity from the blood atoxyl mixture, but the relative amounts of it present in different solutions may be estimated by seeing to what extent these solutions may be diluted before the toxic influence on the trypanosomes disappears.

While working in 1910 in the George Speyer House, Frankfurt a/M., on a problem suggested by Professor Ehrlich, it became desirable to discover whether the strength of the atoxyl which was incubated with blood had any influence on the amount of transformed atoxyl produced. A number of different strengths were tested, first in Frankfurt, and later at The Rockefeller Institute, but most of my experiments were with 1 per cent. and 10 per cent. atoxyl in blood, and it is the results with these two strengths that will be reported in this paper. Moreover, as the technique in the later experiments yielded results that were somewhat easier to tabulate than those obtained in the earlier experiments, the tables given in this paper will be taken exclusively from the experiments done at The Rockefeller Institute. The results reported are, however, in exact accord with those obtained by me in the George Speyer House.

\* Received for publication, December 30, 1914.



TECHNIQUE.<sup>1</sup>

The atoxyl was dissolved in fresh rabbit blood to make 1 and 10 per cent. solutions, respectively. These solutions in blood were incubated at 37° C. for 1 to 3 hours, diluted with salt solution (0.9 per cent.), so as to give, after the addition of 0.5 c.c. of a standard trypanosome suspension to each 0.5 c.c. of dilution, end dilutions of 1:500, 1,000, 2,000, 4,000, 8,000, and 16,000.

It should be noted that these end dilutions contained some unaltered atoxyl and varying numbers of rabbit corpuscles, the number decreasing as the dilution increased. The corpuscles were usually not removed as the rest of the experiment was of short duration and was to be carried out at room temperature. Under these conditions it was thought that the small number of corpuscles present would at this temperature have a negligible influence on the unchanged atoxyl. Whether or not this assumption was justified will be seen in the results reported in this paper.

The adding of the trypanosomes to each of these dilutions of transformed atoxyl was so timed that the examination of each dilution could be made after exactly 30, 60, and 90 minutes' contact with these parasites. The influence which the dilution exerted on the trypanosomes was then recorded in terms of motility of the parasites, this being reckoned on a per cent. basis. 100 per cent. indicates that the trypanosomes were perfectly active, 0 per cent. that all of them had been immobilized. Intermediate influences were indicated by intermediate percentages.

*The Tables.*—In interpreting the results in the tables we should recall:

1. That the dilutions of atoxyl in the tables are comparable, for they represent in all cases the number of times one part of atoxyl has been diluted. To obtain these dilutions the 10 per cent. solution was in each instance diluted ten times as much as the 1 per cent. solution.

2. The motility per cent. of the trypanosomes is in inverse ratio to the amount of free toxin present in the dilution. Great motility of the trypanosomes indicates the absence of much toxicity, but little or no motility shows the presence of much toxin.

3. To make the comparison of the results more striking the figures which indicate a greater production of toxin by one solution than by the other are printed in heavier type.

*Incubation for One Hour.*—When 1 per cent. atoxyl in blood and 10 per cent. atoxyl in blood were incubated at 37° C. for 1 hour it was at once obvious that the 10 per cent. atoxyl solution produced distinctly more than 10 times as much free toxin (transformed atoxyl) as the 1 per cent. atoxyl solution. This result has been obtained many times and three typical experiments showing it are recorded in table I.

<sup>1</sup> The technique of my experiments with atoxyl is given in greater detail in a paper entitled: *The Effect of Heat on the Transforming and Binding Power of Blood* (Terry, B. T., *Jour. Exper. Med.*, 1915, xxi, 267).

TABLE I.

No.	Method.	Dilution 4,000. Motility per cent.	Dilution 2,000. Motility per cent.	Dilution 1,000. Motility per cent.	After contact for min.
9.100	A (1%, 37°, 1 hr.)	100	100	100	30
		100	100	100	60
		100	100	50	90
9.101	A (10%, 37°, 1 hr.)	100	40	20	30
		30	10	0	60
		0	0	0	90
9.114	A (1%, 37°, 1 hr.)	100	100	100	30
		100	100	100	60
		100	100	60	90
9.115	A (10%, 37°, 1 hr.)	100	50	10	30
		50	20	0	60
		20	0	0	90
9.134	A (1%, 37°, 1 hr.)	100	100	100	30
		100	100	100	60
		100	30	5	90
9.135	A (10%, 37°, 1 hr.)	100	30	0	30
		30	0	0	60
		0	0	0	90

In table I we observe that the motility of the trypanosomes was but little affected in the dilutions from the 1 per cent. atoxyl. In these dilutions after contact for 30 and 60 minutes no effect was seen, but after contact for 90 minutes an effect was observed in four instances, three times in the dilution 1 to 1,000, and once in the dilution of 1 to 2,000.

In striking contrast was the effect produced by the dilutions made from the 10 per cent. atoxyl. In dilutions of 1 part in 1,000 and 1 in 2,000 the motility was decreased markedly after a contact of 30 minutes, and in two of the three experiments the trypanosomes were completely immobilized after 90 minutes' contact with dilutions of 1 part in 4,000. If we look at the figures printed in heavier type we see that in twenty-four instances out of a possible twenty-seven, the dilutions of the 10 per cent. atoxyl showed more toxicity than the corresponding dilutions of the 1 per cent. atoxyl. In the remaining three instances on account of the greatness of the dilution (1 to 4,000) and the shortness of the contact (30 minutes) no perceptible effect on the trypanosomes was seen in the dilutions either of the 1 per cent. or of the 10 per cent. solutions.

*Incubation for Three Hours.*—Where the incubation of the 1

and 10 per cent. atoxyl solutions in blood was for 3 hours instead of for 1, the result was quite different, as will be seen in table II, in which the results of three experiments are recorded. Some of the dilutions of the 10 per cent. solution continued to be more toxic than those of the 1 per cent. solution, but the differences in the toxicity of the dilutions was no longer great. In seven instances the dilutions of the 10 per cent. solution were more toxic than those of the 1 per cent. solution, but in one instance a dilution, 8,000, of the 1 per cent. solution was more toxic than the corresponding dilution of the 10 per cent. solution.

TABLE II.

No.	Method.	Dilution 8,000. Motility per cent.	Dilution 4,000. Motility per cent.	Dilution 2,000. Motility per cent.	After contact for min.
9.96	A (1%, 37°, 3 hrs.)	100	100	60	30
		50	10	0	60
		10	0	0	90
9.97	A (10%, 37°, 3 hrs.)	100	40	20	30
		50	10	0	60
		10	0	0	90
9.118	A (1%, 37°, 3 hrs.)	100	100	50	30
		50	20	0	60
		20	0	0	90
9.119	A (10%, 37°, 3 hrs.)	100	80	20	30
		50	20	0	60
		40	0	0	90
9.138	A (1%, 37°, 3 hrs.)	100	80	20	30
		30	10	0	60
		0	0	0	90
9.139	A (10%, 37°, 3 hrs.)	100	20	0	30
		30	0	0	60
		0	0	0	90

If we examine table II more closely we note that of the seven instances in which the dilutions of the 10 per cent. atoxyl were more toxic than those of the 1 per cent., six were seen where the contact with the trypanosomes was shortest; *i. e.*, 30 minutes; that the seventh instance was seen after a contact of 60 minutes; and that the one instance in which a dilution of the 1 per cent. was more toxic than the corresponding dilution of the 10 per cent. solution, was observed after a contact of 90 minutes.

These observations suggested that the 10 per cent. dilutions were probably more toxic immediately after incubation than the 1 per cent. dilutions, but that the 1 per cent. dilutions became correspondingly more and more toxic the longer they were allowed to stand. If this were really the case the increased toxicity was probably due to the red blood corpuscles continuing to act after the incuba-

tion was over. This explanation seemed the more probable because the number of red blood corpuscles in the dilutions of the 1 per cent. solution was ten times as great as those in the 10 per cent. solution.

It was easy to test the influence which the red blood corpuscles might exert after incubation, for the corpuscles could be removed at once by centrifugalization and the corpuscle-free solutions could be tested as before and the figures compared. This was done and the results after 3 hours' incubation are seen in table III.

TABLE III.

No.	Method.		Dilution 8,000. Motility per cent.	Dilution 4,000. Motility per cent.	Dilution 2,000. Motility per cent.	After con- tact for min.
9.98	A (1%, 37°, 3 hrs.)	CC	100	100	100	30
			100	100	60	60
			100	70	10	90
9.99	A (10%, 37°, 3 hrs.)	CC	100	40	20	30
			60	10	5	60
			10	0	0	90
9.120	A (1%, 37°, 3 hrs.)	CC	100	100	100	30
			100	100	100	60
			100	100	70	90
9.121	A (10%, 37°, 3 hrs.)	CC	100	80	10	30
			80	10	0	60
			20	0	0	90
9.140	A (1%, 37°, 3 hrs.)	CC	100	100	100	30
			100	100	30	60
			100	80	0	90
9.141	A (10%, 37°, 3 hrs.)	CC	100	30	5	30
			30	0	0	60
			30	0	0	90

CC means clear centrifugalized fluid containing transformed atoxyl but no corpuscles.

In table III we note that after 3 hours' incubation the 10 per cent. atoxyl dilutions are as much more toxic than the 1 per cent. dilutions, as they were found to be in table I when the incubation period was only 1 hour. In both table I and table III the 10 per cent. solutions were in twenty-four instances out of a possible twenty-seven decidedly more toxic than the corresponding dilutions of the 1 per cent. solution, and in both tables in three instances the effect on the trypanosomes was too slight to be detected.

As the results in table II differ markedly from those in table III, and as the removal of the corpuscles by centrifugalization is the only difference in the technique, we conclude that it was the corpuscles in table II which led to the relatively increased toxicity seen in the dilution of the 1 per cent. atoxyl.

If the presence of corpuscles could give misleading results when the incubation period was 3 hours, it was possible that they might do the same when the incubation was for 1 hour. It became desirable therefore to repeat the experiments recorded in table I, but this time removing the corpuscles by centrifugalization immediately after incubation. This was done and the results obtained in this way are recorded in table IV.

TABLE IV.

No.	Method.		Dilution 2,000. Motility per cent.	Dilution 1,000. Motility per cent.	Dilution 500. Motility per cent.	After con- tact for min.
9.102	A (1%, 37°, 1 hr.)	CC	100	100	100	30
			100	100	100	60
			100	100	100	90
9.103	A (10%, 37°, 1 hr.)	CC	100	80	20	30
			60	0	0	60
			30	0	0	90
9.116	A (1%, 37°, 1 hr.)	CC	100	100	100	30
			100	100	100	60
			100	100	100	90
9.117	A (10%, 37°, 1 hr.)	CC	100	70	5	30
			70	10	0	60
			30	0	0	90
9.136	A (1%, 37°, 1 hr.)	CC	100	100	100	30
			100	100	100	60
			100	100	100	90
9.137	A (10%, 37°, 1 hr.)	CC	100	30	0	30
			30	0	0	60
			10	0	0	90

Table IV shows plainly that after incubation for one hour and in the absence of all corpuscles the dilutions of the 10 per cent. atoxyl were much more than 10 times as toxic as the corresponding dilutions of the 1 per cent. atoxyl. We note that even after 90 minutes' contact with trypanosomes none of the dilutions of the 1 per cent. atoxyl showed the slightest evidence of toxicity in the lowest dilution examined, 1:500, whereas in all three experiments with 10 per cent. atoxyl the parasites were completely immobilized in 90 minutes in dilutions of 1:1,000.

It is evident from the experiments here reported that the strength of the atoxyl solution in blood may have a marked effect on the result. If it is desired to produce a large quantity of transformed atoxyl by incubating with blood for only 1 to 3 hours at 37° C., a strong solution of atoxyl (10 per cent.) should be chosen in preference to a weaker one (1 per cent.).

*Suggested Explanation.*—It may not be out of place to suggest at this point the explanation which seems to me to account most satisfactorily for the results here reported even though this explanation cannot be said to have been proved in every particular and is based in part on experiments not recorded in this paper. It is as follows: Red blood cells transform atoxyl and have a strong affinity for the transformed atoxyl. They unite, therefore, with transformed atoxyl, the quantity with which they can unite being considerable but having definite limits. Not until these limits have been passed does the fluid in which the corpuscles are suspended become toxic for trypanosomes, for the union of the red blood corpuscles and the transformed atoxyl seems to be firm. Blood that has bound as much transformed atoxyl as it can, continues for a time at least to transform atoxyl, and this transformation takes place even at room temperature, although it is faster at 37° C.

*Application to Tables I and IV.*—The small toxicity shown by the dilutions of 1 per cent. atoxyl is due to the fact that in 1 hour's incubation at 37° not quite enough transformed atoxyl is produced to satisfy the red blood cells. If, however, these cells are not removed from the dilutions (table I) they continue to transform the atoxyl during the time the dilutions are acting on the trypanosomes, so that after 90 minutes a little free toxin is present. In the case of the 10 per cent. solutions the transformation goes on more rapidly, and the point at which the corpuscles cease to take up transformed atoxyl is reached sooner. Thereafter the transformed atoxyl remains in solution and acts upon the trypanosomes. By the end of the first hour there is considerable free toxin in solution. As the 10 per cent. solution produces more toxin than the 1 per cent. solution, and as all, or nearly all, the toxin produced by the 1 per cent. solution is taken up by the corpuscles, the 10 per cent. solution appears to have produced many times as much transformed atoxyl as the 1 per cent., although in reality it may not have produced quite ten times as much.

This explanation should also be borne in mind when atoxyl is used in treatment of trypanosome infections. If a small dose is given it is quite possible that all the atoxyl transformed will be taken up by the cells of the host and that none will be available for

the trypanosomes. On the other hand, a large single dose may prove very much more toxic for the host than two half doses, for if a single large dose is given, the cells of the body probably bind what they can of the toxin they produce and the rest is free to poison the trypanosomes and cells of the host that may have an affinity for the transformed atoxyl. If the dose is divided and given on different days, the host may on the first day transform half as much as if the full dose were given; but since the body cells must be satisfied, the amount of free toxin present will not be half of the free toxin produced by the full dose. In 24 hours we assume that much or all of the transformed atoxyl will be bound or excreted. When, therefore, the second half dose is given, free toxin is again produced, but much of this will probably be bound by the body cells before any will be free in fluids which have already lost most or all of the toxin produced when the first half dose was given.

In this connection Koch's<sup>2</sup> experience in Africa may be referred to. At one time Koch injected into his sleeping sickness patients doses varying from 0.5 of a gram to 1 gram. In some of the patients that received the larger doses Koch observed a symptom he had never seen in patients that had received only 0.5 of a gram. Some of those receiving the larger doses became permanently blind in both eyes. As soon as Koch was convinced that the blindness was due to the large doses, he reduced these to 0.5 of a gram given on two succeeding days, and he states that no further cases of blindness resulted.

Before concluding the paper one other point should be mentioned. The four tables show that leaving the corpuscles in with the atoxyl and transformed atoxyl made a great difference in the result when the dilutions were from the 1 per cent. atoxyl in blood. On the other hand, the presence of corpuscles in the dilutions made from the 10 per cent. atoxyl in blood did not alter the result materially. This, of course, is due to the fact that the corpuscles were 10 times as numerous in the dilutions from the 1 per cent. atoxyl as they were in the dilutions from the 10 per cent. atoxyl.

<sup>2</sup> Koch, R., *Deutsch. med. W'chenschr.*, 1907, xxxiii, 1889.

## SUMMARY.

1. 10 per cent. atoxyl in blood incubated at  $37^{\circ}$  C. for 1 hour gives rise to a solution that is much more than ten times as toxic as a 1 per cent. solution of atoxyl similarly incubated.

2. When the comparison is made after incubation for 3 hours instead of for 1 hour, the toxicity of the 10 per cent. solution is but slightly greater than ten times that of the 1 per cent., provided the red blood corpuscles are not removed from the dilutions.

3. If the corpuscles are removed from both the 10 per cent. and the 1 per cent. atoxyl solutions immediately after incubation at  $37^{\circ}$  for 1 to 3 hours, the dilutions of the 10 per cent. atoxyl are much more than ten times as toxic as the corresponding dilutions of the 1 per cent. atoxyl.

4. After incubation with atoxyl at  $37^{\circ}$  for 1 to 3 hours, red blood corpuscles left at room temperature in dilutions made from the 10 per cent. and 1 per cent. solutions in blood increase markedly the toxicity of the dilutions made from the 1 per cent. atoxyl, but increase very slightly the toxicity of the dilutions made from the 10 per cent. atoxyl.

5. If one desires to produce a large amount of transformed atoxyl by incubating atoxyl in blood at  $37^{\circ}$  for 1 to 3 hours, strong solutions of atoxyl should be chosen in preference to weaker solutions.



# THE EFFECT OF HEAT ON THE TRANSFORMING AND BINDING POWER OF BLOOD.\*<sup>1</sup>

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## INTRODUCTION.

In 1910 Yamanouchi published two papers<sup>2, 3</sup> on the action of atoxyl. In these papers he pointed out that red blood corpuscles incubated with a 4 per cent. solution of atoxyl for 2½ hours at 38° C. transform this medicament into a trypanocidal substance. This transforming power Yamanouchi found to be thermolabile, for it was lost when the blood was heated to 80° C. for 30 minutes. The effect on blood of temperatures below 80° C. or of incubation periods shorter or longer than 30 minutes were not reported.

In continuing work at The Rockefeller Institute upon a problem assigned me in the George Speyer House, Frankfurt a/M., by Professor Paul Ehrlich, I had occasion to carry out experiments the results of which are in accord with those of Yamanouchi quoted above. My experiments, however, go further. They establish more exactly the temperature at which the transforming power of blood is lost and show in addition the effect heat has upon the binding power of blood.

## TECHNIQUE.

Defibrinated rabbit blood was used in all the experiments. To obtain transformed atoxyl 1 to 10 per cent. atoxyl was dissolved in blood and the blood was placed in small sterile bottles holding about 2 c.c. each. The bottles were then closed tightly with corks which had been impregnated by immersing for a few seconds in paraffin heated to 200° to 210° C. As soon as the corks cooled they were inserted in the mouths of the bottles and were fastened there securely by means of fine copper wire. The bottles were then immersed in a large quantity of water kept accurately at 37° C. They were shaken vigorously under water for 3 minutes and then were allowed to remain at rest under water for 2 hours

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<sup>1</sup> Some of the points given in detail in this paper were summarized in a paper that appeared in 1913 (Terry, B. T., *Jour. Pharmacol. and Exper. Therap.*, 1913, iv, 361).

<sup>2</sup> Yamanouchi, T., *Compt. rend. Soc. de biol.*, 1910, lxxviii, 120.

<sup>3</sup> Yamanouchi, Ueber die Wirkung des Atoxyls auf Trypanosomen im Organismus, Paris, 1910.

and 57 minutes. The bottles were then removed from the warm water, cooled in tap water, and a number of dilutions were made with sterile 0.9 per cent. salt solution. Small volumes of these dilutions, 0.5 c.c. usually, were then tested by adding to them an equal volume of a trypanosome suspension in serum. *Trypanosoma brucei* (goat normal passage iii), obtained through the kindness of Professor Ehrlich, was used in all these experiments. In making the suspension a richly infected mouse was bled into 10 to 15 c.c. of sterile filtered cattle serum, the serum was centrifugalized sufficiently to throw down the red blood cells, but not the trypanosomes. By means of a blood counter the serum suspension was made to contain the standard number of trypanosomes; *i. e.*, 50 in a volume  $1/4$  by  $1/4$  by  $1/10$  mm. In all instances in which the effect of varying temperatures upon blood was studied, the blood was heated by immersing the bottles containing it in water heated to the requisite temperature. Time was not counted until a thermometer in a control bottle showed that the temperature desired had been reached in this bottle. Before heating blood to temperatures which caused it to coagulate, it was usually diluted to prevent the formation of a firm clot.

In these experiments the thermometer employed for temperatures below 60° C. was very sensitive and agreed closely with a number of other good thermometers, but had not been specially corrected. It registered only 62° C. and each degree was divided in tenths. With a hand lens it was easy to read  $1/20$  of a degree. For temperatures above 60° C. other thermometers that registered higher were employed.

*Recording Motility.*—The effect of the various dilutions on the trypanosomes was recorded in percentages of motility, 100 per cent. meaning that the motility of the organisms was not influenced, 0 per cent. that all the trypanosomes had been immobilized. Intermediate percentages indicate intermediate degrees of influence, these being recorded after referring to a table that I had prepared to facilitate and make uniform the estimation of these percentages. As the influence exerted depended upon the length of time that the toxic solution had been in contact with the trypanosomes, the number of minutes' contact is indicated in each table. The dilutions recorded are in all cases end dilutions; that is, the dilution of atoxyl which resulted after the addition of the trypanosomes. In estimating the dilution no allowance could be made for the atoxyl which was converted into transformed atoxyl during the incubation. For example, the dilution 1:1,000 means that the original 10 per cent. atoxyl in blood was diluted 50 times with salt solution, making an atoxyl dilution of 1:500, and that then 1 volume of this solution was added to an equal volume of a trypanosome suspension, making an end dilution of 1:1,000.

*General Plan.*—In studying the effect of heat upon the transforming power of blood, comparatively low temperatures were first employed. When these were found to have no effect, higher temperatures were tried until temperatures were found which destroyed the transforming power of blood. The interval between the temperatures that had no effect and the temperatures that destroyed

the transforming power were then studied to determine more exactly the lowest temperature that would destroy this power.

*37° and 50° C.*—Blood heated to 37° C. and to 50° C. for half an hour before being incubated with atoxyl showed no perceptible alteration in its transforming power.

TABLE I.

No.	Method.			Dilution 100.		Dilution 300.		Dilution 1,000.		Dilution 3,000.	
	Transforming agent.	Preliminary heat.	Incubation.	Motility per cent.	Min.	Motility per cent.	Min.	Motility per cent.	Min.	Motility per cent.	Min.
5.84	Blood	37°, 30 min.	A (5%, 37°, 3 hrs.)	0	18	0	26	0	55	0	84
5.85	Blood	50°, 30 min.	A (5%, 37°, 3 hrs.)	0	18	0	26	0	55	0	84
5.90 to 5.95	Blood	50°, 30 min.	A (5%, 37°, 3 hrs.)	0	16	0	22	0	66	8	70
5.90 to 5.95	Blood	No preliminary heat	A (5%, 37°, 3 hrs.)	0	16	0	22	0	66	8	70

A is the abbreviation for atoxyl.

From table I we see that a sample of blood heated to 37° for 30 minutes behaved exactly like a sample heated to 50° for 30 minutes; and another sample heated to 50° for 30 minutes behaved like a sample that had not been heated at all. The trypanosomes were completely immobilized in all dilutions except the one corresponding to 1 part of atoxyl in 3,000. Here after 70 minutes the trypanosomes in two samples were 8 per cent. motile. In two other samples of the same dilution all the trypanosomes were immobilized after a contact of 84 minutes.

*55° C. and Higher Temperatures.*—Blood heated to 55° C., 57° C., and 60° C. for 30 minutes before it was incubated with atoxyl lost most of its transforming power. This is shown in table II.

In table II we see that blood heated preliminarily to 55° C., 57° C., and 60° C. transformed atoxyl so little when subsequently incubated with it that in a dilution of 1 in 1,000 no action was seen on trypanosomes even after 66 to 86 minutes' contact. On the other hand, the transforming power of the blood that was not heated preliminarily was so great that all the trypanosomes were immobilized in dilutions of atoxyl equal to 1 in 1,000, and in the dilution 1 in 3,000 the organisms were only 8 per cent. motile after 70 minutes' contact. We note in the table, however, that the heated blood did not lose all its transforming power, for we see that the trypanosomes were distinctly influenced in the stronger dilutions. For example, the trypanosomes were 28 to 51 per cent. motile in two dilutions of atoxyl corresponding to 1 part in 300, and in the stronger dilutions of 1 in 100 the influence of the residual transforming power was still more obvious.

TABLE II.

No.	Method.			Dilution 100.		Dilution 300.		Dilution 1,000.		Dilution 3,000.	
	Transforming agent.	Preliminary heat.	Incubation.	Motility per cent.	Min.	Motility per cent.	Min.	Motility per cent.	Min.	Motility per cent.	Min.
5.86	Blood	55°, 30 min.	A (5%, 37°, 3 hrs.)	8	80	100	85	100	86	100	88
5.90 to 5.95	Blood	55°, 30 min.	A (5%, 37°, 3 hrs.)	8	50	51	60	100	66	100	70
5.90 to 5.95	Blood	57°, 30 min.	A (5%, 37°, 3 hrs.)	3	63	28	64	100	66	100	72
5.87	Blood	60°, 30 min.	A (5%, 37°, 3 hrs.)	0	80	100	85	100	86	100	88
5.90 to 5.95	Blood	Control not heated preliminarily	A (5%, 37°, 3 hrs.)	0	50	0	60	0	66	8	70

The interval between 50° C. and 55° C. was next studied. Blood was heated at 51° C., 53° C., and 55° C. for 30 minutes, and at 55° C. also for 15 minutes and for 7 minutes. All samples, including one that had not been heated at all, were then tested for their transforming power by making in each a 5 per cent. solution of atoxyl and incubating for 3 hours at 37°. The result of this experiment is shown in table III.

TABLE III.

No.	Method.			Dilution 500.		Dilution 1,000.		Dilution 2,000.		Dilution 4,000.	
	Transforming agent.	Preliminary heat.	Incubation.	Motility per cent.	Min.	Motility per cent.	Min.	Motility per cent.	Min.	Motility per cent.	Min.
5.102 to 5.105	Blood	51°, 30 min.	A (5%, 37°, 3 hrs.)	0	39	0	52	4	54	58	60
5.102 to 5.105	Blood	55°, 7 min.	A (5%, 37°, 3 hrs.)	0	39	14	54	4	54	84	60
5.102 to 5.105	Blood	53°, 30 min.	A (5%, 37°, 3 hrs.)	0	39	7	54	16	54	93	60
5.102 to 5.105	Blood	55°, 15 min.	A (5%, 37°, 3 hrs.)	0	39	25	54	72	54	100	60
5.102 to 5.105	Blood	55°, 30 min.	A (5%, 37°, 3 hrs.)	100	39	100	53	100	54	100	60
5.102 to 5.105	Blood Control	None	A (5%, 37°, 3 hrs.)	0	39	7	52	2	54	48	60

We see in table III that 51° C. for 30 minutes had scarcely a perceptible effect on the transforming power, for the result resembles very closely that of the unheated control blood. We note also that 55° for 7 minutes had a slight effect; that 53° for 30 minutes was more injurious; that 55° for 15 minutes was still more injurious; and that 55° for 30 minutes was the most injurious of all.

In the experiment just described the greatest change caused by heating for 30 minutes was produced by temperatures between 53° C. and 55° C. This interval was therefore studied again, the heating being prolonged in some instances to 60 minutes.

It was found that 53° for one hour apparently destroyed the transforming power of blood completely, but heating at 53° for 30 minutes was much less effective. Between 53° and 54° for 60 minutes and 55° for 30 minutes no difference was seen.

In another experiment 53° for 60 minutes destroyed almost but not quite all the transforming power of blood.

We may summarize the effect of the various temperatures and intervals thus far studied as follows: when the heating was for 60 minutes, 54° C. seemed to destroy all the transforming power; but when the heating was for 30 minutes, 55° C. apparently destroyed nearly all this power; 52° to 54° C., correspondingly less; 51°, almost none; and 50°, none.

*Higher Temperatures.*—Temperatures of 70° and 100° destroyed the transforming power of blood more rapidly, as will be seen in table IV.

TABLE IV.

No.	Method.				Dilution 40.		Dilution 120.		Dilution 360.		Dilution 3,240.	
	Trans- forming agent.	Dilu- tion before heat- ing.	Preliminary heat.	Incubation.	Motility per cent.	Min.	Motility per cent.	Min.	Motility per cent.	Min.	Motility per cent.	Min.
7.65	Blood	10	70°, 10 min.	A (5%, 37°, 3 hrs.)	23	117	89	117	100	118	100	113
7.66	Blood	0	100°, 10 min.	A (5%, 37°, 3 hrs.)	Too little to test		100	122	100	122	100	120
7.67	Blood	10	100°, 10 min.	A (5%, 37°, 3 hrs.)	100	128	100	128	100	128	100	124
7.68	Blood	10	No heat	A (5%, 37°, 3 hrs.)	0	40	0	40	0	41	0	41

Blood heated preliminarily to 70° C. for 10 minutes lost nearly all its transforming power, and blood heated to 100° for 10 minutes seemingly lost all its transforming power. This result was obtained repeatedly.

## HEAT SUBSEQUENT TO INCUBATION.

The experiments thus far recorded have dealt with the effect on blood of heat applied before the blood had been incubated with atoxyl. Other experiments were made to determine the effect of heat on blood after it had been incubated with atoxyl, and in the course of these experiments several interesting facts were revealed, for it was found that as the heat increased the toxicity of the transformed atoxyl for trypanosomes varied in an unexpected manner. With lower temperatures the toxicity increased as the temperature was raised. When certain temperatures were reached, however, the toxicity began to decrease, and at one temperature disappeared almost completely. As the temperature was raised still higher, however, the toxicity began to return, and at 100° C. the solution was almost as toxic as it had been immediately after the initial incubation at 37° for 3 hours. The experiments showing these changes will now be given.

Rabbit blood was incubated with 10 per cent. atoxyl for 3 hours at 37° C. It was then diluted five times with salt solution to prevent the formation of firm clots during the subsequent heating, and was divided into a number of samples. One of them, kept as a control, was heated no further. The other samples were heated for 10 minutes each to a different temperature, 50°, 56°, 60°, 70°, 80°, 90°, and 100° C. Each sample was then diluted so that its comparative toxicity for trypanosomes could be determined. The result is shown in table V.

From table V we see that heating the blood to 50° for 10 minutes subsequent to incubation with atoxyl increased its toxicity for trypanosomes, for in the atoxyl dilution of 1 part in 8,100 the trypanosomes were 20 per cent. motile after 60 minutes' contact, and only 10 per cent. motile after 90 minutes' contact. In the same dilution of unheated blood the trypanosomes were 100 per cent. motile after 60 minutes' and 20 per cent. motile after 90 minutes' contact.

We see also in table V that the blood heated to 56° C. was more toxic than that heated to 50° C., but that the sample heated to 60° C. for 10 minutes was less toxic, being about as toxic as the sample heated to 50° for 10 minutes.

TABLE V.

No.	Method of transforming atoxyl.	Subsequent heat.	Dilution 8,100.		Dilution 2,700.		Dilution 900.		Dilution 300.	
			Motility per cent.	Min.	Motility per cent.	Min.	Motility per cent.	Min.	Motility per cent.	Min.
9.54	Blood + A (10%, 37°, 3 hrs.)	None	100	30	60	30	0	30	0	30
			100	60	0	60	0	60	0	60
			20	90						
9.55	Blood + A (10%, 37°, 3 hrs.)	50°, 10 min.	100	30	20	30	0	30	0	30
			20	60	0	60	0	60	0	60
			10	90						
9.56	Blood + A (10%, 37°, 3 hrs.)	56°, 10 min.	50	30	0	30	0	30	0	30
			10	60	0	60	0	60	0	60
			0	90						
9.57	Blood + A (10%, 37°, 3 hrs.)	60°, 10 min.	100	30	10	30	0	30	0	30
			30	60	0	60	0	60	0	60
			10	90						
9.58	Blood + A (10%, 37°, 3 hrs.)	70°, 10 min.	100	30	100	30	100	30	100	30
			100	60	100	60	100	60	100	60
			100	90						
9.59	Blood + A (10%, 37°, 3 hrs.)	80°, 10 min.	100	30	100	30	100	30	100	30
			100	60	100	60	100	60	80	60
			100	90						
9.60	Blood + A (10%, 37°, 3 hrs.)	90°, 10 min.	100	30	100	30	100	30	5	30
			100	60	100	60	20	60	0	60
			100	90						
9.61	Blood + A (10%, 37°, 3 hrs.)	100°, 10 min.	100	30	100	30	50	30	0	30
			100	60	80	60	0	60	0	60
			100	90						

In table V the decrease in toxicity caused by heating the blood subsequent to incubation is strikingly shown after heating to 70° C., for the trypanosomes brought in contact with the sample heated to this temperature for 10 minutes remained 100 per cent. motile even after contact for 60 minutes in a dilution of atoxyl corresponding to 1 in 300.

Heating to 80° C. left the sample a little more toxic than heating it to 70° C., for we note in the atoxyl dilution of 1 in 300 that after 60 minutes' contact the trypanosomes were but 80 per cent. motile instead of 100. Heating to 80° C. caused, therefore, the return of a slight amount of toxicity. Heating to 90° C. caused the return of more of the toxicity, and heating to 100° C. caused the return of still more; for we see in table V that after heating to 100° C. for 10 minutes all the parasites were immobilized in an atoxyl dilution of 1 in 900 after contact for 60 minutes.

The effects in table V which interested me most were produced by heating to 70° C. and to 100° C. These temperatures were employed, therefore, in a number of similar experiments, and in all of them it was found that heating to 70° C. caused the toxic substance to decrease greatly, while heating to 100° C. caused the toxicity of the solution in blood to return more or less completely.

*Suggested Explanation.*—Blood incubated at 37° C. with 1 to 10 per cent. atoxyl transforms this medicament in part into a trypanocidal substance. Some of the trypanocidal substance is bound by the red blood cells (this seems evident from experiments not reported in this paper), but some of it remains free and is detected when trypanosomes are added to the solution. When blood is incubated with atoxyl at 37° C. a double process goes on; atoxyl is transformed and transformed atoxyl is bound. The double process continues as the temperature is raised, but in most experiments the transforming process is the more prominent, for an increase in toxicity is noted as the temperature is raised. When the temperature reaches 54° to 56° C., however, the transforming process soon ceases and at 60° C. the binding process makes itself evident.

At about 70° C. the binding process reaches its maximum and the blood takes up most of the toxin in solution. At higher temperatures (or, as we shall soon see, on prolonged heating at 70° C.) a third, or liberating, process begins, the transforming and binding processes being no longer operative. The blood therefore begins to give up the toxin it has bound, and at 100° C. most of the toxin is free again.

*Prolonged Heating.*—When blood that had been incubated at 37° C. for 3 hours with atoxyl was subsequently heated to 70° and to 100° for 30 minutes instead of for 10 minutes, it was found that both samples were more toxic than similar samples heated for 10 minutes. Even at 70° the prolonged heating evidently liberated some of the toxin and at 100° C. for 30 minutes, freed all or nearly all the toxin.

As controls on the experiments in which blood incubated with atoxyl was subsequently heated to 100° for 10 and for 30 minutes, blood alone and atoxyl alone were heated to 100° for 10 and for 30 minutes and tested with trypanosomes. These experiments showed that the heating separately of blood and atoxyl to 100° for 10 and for 30 minutes did not make either of them toxic.

The power of blood to take up and bind transformed atoxyl on being heated to 70° C. for 10 minutes in its presence is apparently completely lost when blood is heated to 100° for 10 minutes. In one experiment blood that had been incubated with 10 per cent.



atoxyl at  $37^{\circ}$  for 3 hours was diluted five times with salt solution. One sample of this was heated first to  $100^{\circ}$  for 10 minutes, and soon afterwards was heated to  $70^{\circ}$  for 10 minutes. The toxicity of this heated sample and the toxicity of an unheated sample are shown in table VI.

TABLE VI.

Method,						Dilution 8,100.		Dilution 2,700.		Dilution 900.	
						Motility per cent.	Min.	Motility per cent.	Min.	Motility per cent.	Min.
Blood	A (10%, $37^{\circ}$ , 3 hrs.)	Dilution 1 : 5	100°, 10 min.	70°, 10 min.	CC	100	30	20	30	10	30
						100	60	5	60	0	60
						60	90	5	90	0	90
Blood	A (10%, $37^{\circ}$ , 3 hrs.)	Dilution 1 : 5	Not heated subsequently	Not heated subsequently	CC	100	30	10	30	0	30
						50	60	5	60	0	60
						30	90	0	90	0	90

In this and the following tables CC means clear centrifugalized fluid containing transformed atoxyl but no corpuscles.

From table VI we see that the sample heated subsequently to  $100^{\circ}$  for 10 minutes and then to  $70^{\circ}$  for 10 minutes was almost as toxic as the unheated sample. The slight difference is probably due to the fact that heating to  $100^{\circ}$  for 10 minutes does not usually suffice to free all the toxic substance.

*Transformed Atoxyl Enters Corpuscles.*—Transformed atoxyl produced by incubating blood with atoxyl at  $37^{\circ}$  for 3 hours enters the blood corpuscles when the blood is heated in its presence to  $70^{\circ}$  for 10 minutes. This is shown by the fact that on heating the blood and then centrifugalizing it the fluid above the corpuscles loses its toxicity and does not regain it if it is separated from the corpuscles and is then heated to  $100^{\circ}$  for 20 minutes. The corpuscles, on the other hand, if brought into contact with fresh salt solution, make this quite toxic for trypanosomes, if while in contact with it they are heated to  $100^{\circ}$  for 20 minutes. In table VII 1 per cent. atoxyl was used instead of 10 per cent. The reason will soon be apparent.

TABLE VII.

No.	Method.							Dilution 3,000.		Dilution 1,000.	
								Motility per cent.	Min.	Motility per cent.	Min.
9.39	Blood	A (1%, 37°, 3 hrs.)	Dilution 1 : 5	70°, 10 min.	CSS	100°, 20 min.	CC	100 20 10	30 60 90	5 0 0	30 60 90
9.38	Blood	A (1%, 37°, 3 hrs.)	Dilution 1 : 5	70°, 10 min.	CC	100°, 20 min.	CC	100 100 100	30 60 90	100 100 100	30 60 90

CSS means corpuscles suspended in salt solution after being separated from the solution of transformed atoxyl.

From table VII we see that the corpuscles heated to 100° C. for 20 minutes rendered the salt solution quite toxic, for all of the trypanosomes were immobilized in 60 minutes in an atoxyl dilution of 1 to 1,000, and in 90 minutes were almost immobilized (10 per cent.) in a dilution of 1 in 3,000. On the other hand, the supernatant fluid remained non-toxic in the same dilutions.

*Laked Blood Binds Transformed Atoxyl.*—The reason that 1 per cent. atoxyl was used instead of 10 per cent. in the last experiment is that blood is partially laked during its incubation at 37° C. for 3 hours with 10 per cent. atoxyl, and under the influence of the proper temperature laked blood, like unlaked blood, can be made to take up, or to free, transformed atoxyl. This is shown in table VIII.

TABLE VIII.

No.	Method.					Dilution 2,700.	Dilution 900.	Dilution 300.	Dilution 100.	After contact for min.
						Motility per cent.	Motility per cent.	Motility per cent.	Motility per cent.	
8.184	Blood	A (10%, 37°, 3 hrs.)	Dilution 1 : 5	CC	Control not subsequently heated	40 0 0	0 0 0	0 0 0	0 0 0	30 60 90
8.190	Blood	A (10%, 37°, 3 hrs.)	Dilution 1 : 5	CC	70°, 10 min.	100 100 100	100 100 100	100 100 20	100 100 20	30 60 90
8.191	Blood	A (10%, 37°, 3 hrs.)	Dilution 1 : 5	CC	70°, 30 min.	100 100 100	100 100 100	60 10 40	30 5 0	30 60 90
8.192	Blood	A (10%, 37°, 3 hrs.)	Dilution 1 : 5	CC	100°, 10 min.	100 20 0	0 0 0	0 0 0	0 0 0	30 60 90
8.194	Blood	A (10%, 37°, 3 hrs.)	Dilution 1 : 5	CC	100°, 30 min.	10 0 0	0 0 0	0 0 0	0 0 0	30 60 90

From table VIII we see that after partial laking of the red blood corpuscles the supernatant fluid was quite toxic; that heating this fluid to  $70^{\circ}$  for 10 minutes caused nearly all the toxicity to disappear; and that heating it to  $100^{\circ}$  for 10 minutes caused almost all the toxicity to reappear. The solutions heated for 30 minutes were more toxic than those heated for 10 minutes.

*Concentrating the Transformed Atoxyl.*—As transformed atoxyl enters the red blood corpuscles on being heated with them to  $70^{\circ}$  for 10 minutes, it seemed probable that this fact might be utilized to concentrate the toxic substance. In an experiment to test this point blood was first incubated with 5 per cent. atoxyl for 3 hours at  $37^{\circ}$ . It was then diluted  $12\frac{1}{2}$  times with salt solution and one part was heated to  $70^{\circ}$  for 10 minutes, was centrifugalized, the supernatant fluid was poured off, and a small quantity of fresh salt solution (3.5 cubic centimeters) was added to the corpuscles which were then heated to  $100^{\circ}$  for 30 minutes. The supernatant fluid which had been poured off and blood that had not been heated subsequently were tested as controls. The result is shown in table IX.

#### SUMMARY.

1. The transforming power of red blood cells for atoxyl is apparently uninfluenced by a preliminary heating of the blood for 30 minutes at  $50^{\circ}$  C., but a temperature of  $55^{\circ}$  C. acting for 30 minutes destroys this property of blood almost completely.

2. When the heating was for 60 minutes,  $54^{\circ}$  C. and higher temperatures apparently destroyed all the transforming power.

3. After heating for 10 minutes,  $70^{\circ}$  C. destroyed almost all the transforming power, and  $100^{\circ}$  C. seemingly destroyed all.

4. Under the influence of certain temperatures blood seemingly binds transformed atoxyl, and under the influence of other temperatures frees it again. A temperature of  $70^{\circ}$  C. for 10 minutes applied to blood subsequent to its incubation with atoxyl causes nearly all the toxicity of the solution to disappear. On the other hand, a temperature of  $100^{\circ}$  C. for 10 minutes applied to blood subsequent to incubation with atoxyl leaves the solution almost as toxic as if the blood had not been heated at all after the incubation.

5. Some of the transformed atoxyl bound by blood when it is heated to  $70^{\circ}$  C. for 10 minutes is freed if the heating at  $70^{\circ}$  C. is prolonged to 30 minutes.



6. Blood which has bound transformed atoxyl gives up most, but not all, of this toxic substance when heated to  $100^{\circ}$  for 10 minutes. All, or nearly all, is freed when the heating at  $100^{\circ}$  C. is continued for 30 minutes.

7. Under the influence of  $70^{\circ}$  C. for 10 minutes transformed atoxyl may be made to enter blood corpuscles, for the fluid above these corpuscles is rendered non-toxic and does not become toxic again when heated to  $100^{\circ}$  for 20 minutes, provided it contains no laked blood. The blood corpuscles, on the other hand, when heated to  $100^{\circ}$  for 20 minutes in contact with fresh salt solution, make this solution quite toxic.

8. Laked blood in contact with transformed atoxyl behaves like unlaked blood when heated to  $70^{\circ}$  C. and to  $100^{\circ}$  C. for 10 to 30 minutes.

9. The toxic substance can be concentrated by heating the blood corpuscles in contact with transformed atoxyl to  $70^{\circ}$  C. for 10 minutes, centrifugalizing, removing a large part of the supernatant fluid, and then heating to  $100^{\circ}$  C. for 30 minutes.

10. The power of blood to take up and bind transformed atoxyl is destroyed apparently completely by heating blood to  $100^{\circ}$  C. for 10 minutes.

11. The toxic substance into which atoxyl is transformed (transformed atoxyl) is thermostabile, but the transforming agent in blood is thermolabile.

# THE INFLUENCE OF THE ANTERIOR LOBE OF THE PITUITARY BODY UPON THE GROWTH OF CARCINOMATA.\*

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In consequence of the frequently observed correlation between abnormal disturbances of the growth process and pathological conditions in the pituitary body, many investigations have recently been carried out with a view to ascertaining the effect of administrations of the pituitary body or portions thereof upon the time relations and absolute magnitude of normal growth. Especial interest attaches to experiments upon the effects of the anterior lobe upon the growth of young animals, since in cases of acromegaly and gigantism anterior lobe hyperplasia is frequently observed.<sup>1</sup>

The experimental findings in these investigations have not been uniform, possibly on account of the wide variety of methods of administration, experimental animals, etc., employed by different observers. The results which are not purely negative, however, such as those of Caselli,<sup>2</sup> Sandri,<sup>3</sup> and Schäfer,<sup>4</sup> appear to indicate that anterior lobe administration by mouth or hypodermically to young animals causes a notable retardation of growth,<sup>5</sup> while ex-

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<sup>1</sup> Cushing, H., *The Pituitary Body and Its Disorders*, Philadelphia, 1912, 20.

<sup>2</sup> Caselli, A., *Riv. sper. di freniat.*, 1900, xxvi, 176, 468, 486; cited by Cushing, *loc. cit.*

<sup>3</sup> Sandri, O., *Arch. ital. de biol.*, 1909, li, 337.

<sup>4</sup> Schäfer, E. A., *Proc. Roy. Soc., London, Series B*, 1909, lxxxi, 442; *Quart. Jour. Exper. Physiol.*, 1912, v, 203.

<sup>5</sup> Cerletti, U., *Arch. ital. de biol.*, 1907, xlvii, 123. Sandri, O., *loc. cit.* Etienne, G., and Parisot, J., *Arch. de méd. expér. et d'anat. path.*, 1908, xx, 423. Goetsch, E., and Cushing, H., cited by Cushing, *loc. cit.*, p. 11. Aldrich, T. B., *Am. Jour. Physiol.*, 1912, xxx, 352; 1912-13, xxxi, 94. Wulzen, R., *Am. Jour. Physiol.*, 1914, xxxiv, 127.

perimental hypopituitarism in young animals induced by partial extirpation of the anterior lobe produces a series of symptoms which furnish a clinical picture the reverse of acromegaly;<sup>6</sup> namely, retardation of the growth of bones, a pronounced tendency to adiposity, and sexual infantilism.

It appeared to us to be of interest to determine the effects of the administration of anterior lobe upon the growth of carcinomata, both on account of the possibility held out by such an investigation of further confirming and elucidating the relationship of this gland to growth, and also on account of the information which might thus be derived regarding the relationship of carcinomatous to normal growth.

#### HISTORY OF THE TUMORS AND MATERIAL EMPLOYED.

We propagated the Flexner-Jobling carcinoma<sup>7</sup> by inoculation into the axillary region through two generations. The percentage of takes was high, varying between 60 and 80 per cent. Half grown or adult animals were employed to propagate the tumors, and also in the experiments enumerated below.

Through the courtesy of the superintendent, ox pituitaries were obtained from the Oakland Meat and Packing Company. They were dissected out of the connective tissue capsule and the anterior lobe was separated, macerated between two surfaces of ground glass, and mixed with  $\frac{M}{6}$  sodium chloride solution in such proportion that the final volume of the mixture was three times that of the glandular tissue. 0.4 per cent. of tricresol was added and the mixture was shaken and allowed to stand at room temperature for not less than twelve hours. After further shaking the mixture was filtered through glass-wool under pressure. The filtrate was employed in the experiments detailed below; it was freshly prepared from time to time. Three cubic centimeters were equivalent to

<sup>6</sup> Cushing, *loc. cit.*, pp. 13, 27.

<sup>7</sup> Through the courtesy of Dr. Peyton Rous, of The Rockefeller Institute for Medical Research, we obtained specimens of his 21st generation of the Flexner-Jobling tumor early in this year. Dr. Rous's tumor, as he informed us, was not then metastasizing. In our laboratory, however, the first generation of tumors propagated from this specimen metastasized, and succeeding generations have continued to do so (Burnett, T. C., *Proc. Soc. Exper. Biol. and Med.*, 1913, xi, 42).

one cubic centimeter, *i. e.*, to approximately one gram of glandular tissue.

#### EXPERIMENTAL.

*First Series.*—Eighty-five white rats were inoculated in the axillary region with peripheral portions of a large, rapidly growing tumor of our 2d generation from Rous's 21st generation. After 22 days, 28 of the animals (33 per cent.) were found to have well developed tumors. These were divided into two batches of 14 each; one batch was retained as controls, and the individuals comprising the other received, on the 22d, 25th, 28th, 30th, 32d, and 34th days after inoculation, 1.5 c.c. each of pituitary emulsion (0.5 gm. of glandular tissue) injected directly into the tumor.

The tumors were measured through the skin in two diameters at right angles to each other, on the dates enumerated below (table I). The mean of the longest and shortest diameters was recorded as the average diameter of any given tumor, and the average of these estimates is regarded as the average diameter of the tumors in any given group of animals.

The average diameter of the tumors in the control animals on the 22d day after inoculation was 13.9 millimeters; that of the animals reserved for treatment was 14.1 millimeters. Calling these initial diameters in each group 100, and referring each of the subsequent measurements to this unit of comparison, we obtained the following results.

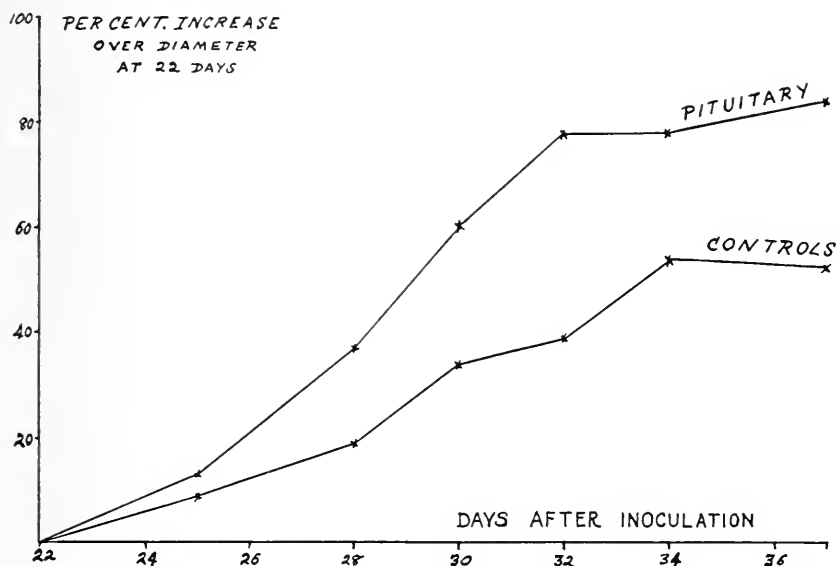
TABLE I.

Dys. after inoculation.	Diameter of tumors in controls.	Diameter of tumors in animals receiving pituitary emulsion.
22.....	100 .....	100
25.....	109 .....	113
28.....	119 .....	137
30.....	134 .....	160
32.....	139 .....	178
34.....	154 .....	178
37.....	152 .....	184

These results are depicted graphically in text-figure 1. It will be seen that the administration of anterior lobe emulsion by injection directly into the tumors caused marked acceleration of the average growth of the primary tumors, their rate of growth after two administrations being no less than double that of the tumors in the control animals. While this was due to an acceleration of the



growth of all the tumors, the acceleration was most marked in the relatively small tumors, with the result that after two or three administrations the treated tumors were all large, while the control tumors were less uniform in size, some being relatively large and others relatively small. Thus on the 32d day, that is, after four



TEXT-FIG. 1. Comparative growth of untreated tumors and tumors treated with pituitary (anterior lobe) emulsion.

administrations of anterior lobe emulsion spread over 10 days, 50 per cent. of the control tumors measured under 20 millimeters in diameter, while only 2, or 14 per cent., of the treated tumors measured 20 millimeters in diameter, and none were smaller than this. The proportion of tumors measuring 25 millimeters or over was, however, the same in both groups, namely 43 per cent., although the average diameter of these large tumors was 31 millimeters in the treated group and only 28 millimeters in the control group.

On the 37th day all the animals were killed and the viscera examined for metastases. Three, or 21 per cent., of the control animals were found to have developed metastases, while none of the treated animals had done so. It appears evident, therefore, that adminis-

trations of the anterior lobe of the pituitary body do not enhance the tendency of carcinomata to form metastases.

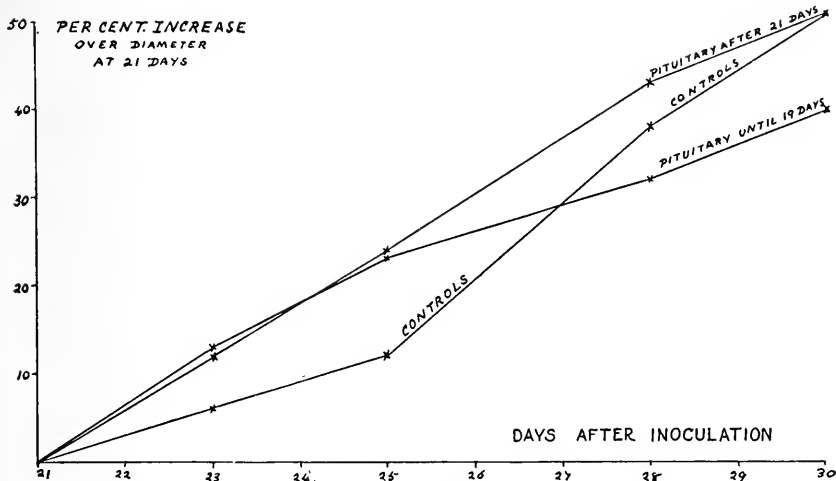
*Second Series.*—In the above experiments the pituitary emulsion was injected directly into the tumors. Fearing that the local conditions created by this procedure might have been responsible for the results obtained, we undertook a second series of experiments.

Sixty white rats were inoculated in the right axillary region with peripheral portions of a large tumor of our 2d generation from Rous's 21st. These were then divided into two lots. Eighteen animals received 1.5 c.c. each of anterior lobe emulsion (0.5 gm. of glandular tissue) on the 7th, 9th, 11th, 14th, 16th, and 19th days after inoculation, the emulsion being injected into the left axillary region. The remaining 42 animals received no treatment of any kind until the 21st day after inoculation, when all the tumors were measured and the percentage of takes was determined.

Of the 18 treated rats, 16, or 89 per cent., had developed tumors. Of the 42 rats not hitherto treated, 35, or 81 per cent., had developed tumors. The average diameter of the tumors in the treated group was 17.9 millimeters, while the average diameter of the tumors in the untreated group was 17.2 millimeters. There was, therefore, no marked acceleration of the tumor growth or increase in the percentage of takes consequent upon the administration of the pituitary emulsion up to the 21st day after inoculation. The administration of the emulsion to the animals hitherto treated was now discontinued, and 34 of the animals which had not been hitherto treated and which had developed tumors were divided into two lots of 17 each. One batch of animals was reserved as controls and not treated in any way; the other received 1.5 cubic centimeters of emulsion (0.5 of a gram of glandular tissue) in the left axillary region on the 21st, 23d, 25th, and 28th days after inoculation. All the tumors were measured on these dates and also on the 30th day after inoculation. The diameter of the tumors on the 21st day in the control animals was 16.4 millimeters, in the formerly treated animals 17.9 millimeters, and in the animals treated subsequent to that date 18.1 millimeters. The relative growth of these three groups of tumors is shown in table II and is depicted graphically in text-figure 2.

As in the previous experiment, the administration of anterior lobe emulsion produced a marked acceleration of the growth of the primary tumor, the rate of growth being doubled after the second

administration. In this experiment, however, the accelerative effect was not long continued and disappeared after the fourth administration on the 30th day after inoculation. It is of especial interest to observe that although the repeated administration of the pitui-



TEXT-FIG. 2. Comparative growth of untreated tumors and of tumors treated with pituitary (anterior lobe) emulsion before and after the twentieth day following inoculation.

tary emulsion prior to the 21st day after inoculation did not appear to influence the growth of the tumor in any notable way, yet some influence had been exerted, or else the active agent of the gland persists in the body for a considerable time after administration, for the previously treated tumors grow as rapidly as the treated tumors from the 21st to the 25th days; that is to say, for a period of 6 days after the discontinuance of the treatments.

TABLE II.

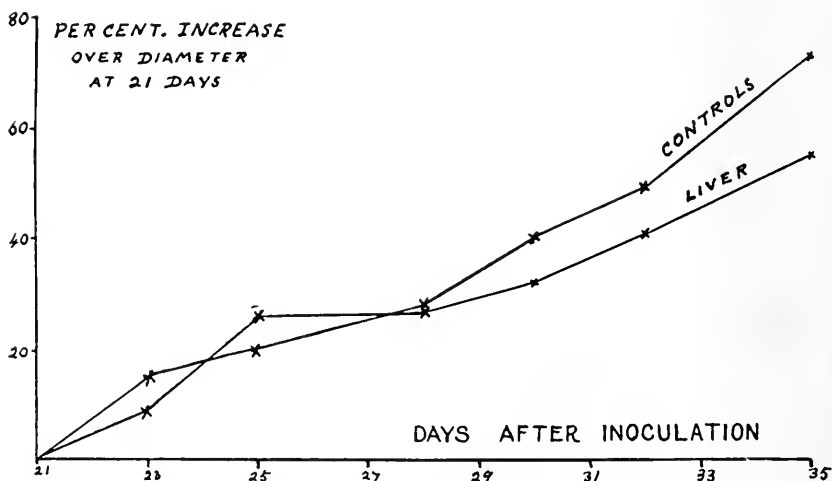
Dys. after inoculation.	Diameter of tumors in controls.	Diameter of tumors in animals receiving pituitary emulsion prior to the 21st dy. after inoculation.	Diameter of tumors in animals receiving pituitary emulsion upon and subsequent to the 21st dy. after inoculation.
21	100	100	100
23	106	113	112
25	112	123	124
28	138	132	143
30	151	140	151

On the 30th day after inoculation all the animals were killed and the viscera examined for metastases. Three animals among the controls were found to have developed metastases, and two animals in each of the treated batches. No evidence was therefore afforded of any effect of the pituitary emulsion upon the tendency of the tumors to form metastases.

#### CONTROL EXPERIMENT WITH LIVER EMULSION.

The above results appeared to justify the conclusion that the hypodermic administration of pituitary (anterior lobe) emulsion to rats markedly accelerates the growth of carcinoma, at least of the primary tumors. We feared, however, that this result might merely be attributable to the administration of tissue emulsion, or to the tricresol employed as the sterilizing agent, and not especially to the employment of pituitary tissue. Accordingly we carried out a series of experiments in which an emulsion of ox liver was employed instead of an emulsion of pituitary gland. The liver emulsion was prepared in exactly the same way as the pituitary emulsion had been prepared.

Fifty-five white rats were inoculated with portions of a tumor taken from one of the controls in the preceding experiment; that is, from our 3d generation



TEXT-FIG. 3. Comparative growth of untreated tumors and of tumors treated with liver emulsion.

propagated from Rous's 21st generation. On the 21st day after inoculation it was found that 34 animals had developed small tumors. These were divided into two batches of 17 each. One batch received 1.5 c.c. of liver emulsion (0.5 gm. of glandular tissue) in the axillary region opposite to the tumor on the 21st, 23d, 25th, 28th, 30th, and 32d days following inoculation. The tumors were measured on the same days. The average diameter of the control tumors on the 21st day was 8.2 mm., that of the treated tumors was 7.4 mm. The relative growth of the control and treated tumors is shown in table III and text-figure 3.

TABLE III.

Dys. after inoculation.	Diameter of tumors in controls.	Diameter of tumors in animals receiving liver emulsion.
21.....	100 .....	100
23.....	115 .....	109
25.....	120 .....	126
28.....	128 .....	127
30.....	140 .....	132
32.....	149 .....	141
35.....	173 .....	155

It is evident that liver emulsion exerts no accelerative action whatever upon the growth of carcinoma; indeed a slight retardation is evident at certain stages. From experiments which we have carried out, the results of which have not yet been published, we are inclined to believe that this retardation may have been attributable to the bile salts contained in the liver extract, as we have observed that bile salts exert a slightly retarding action upon the growth of carcinomata in rats.

#### CONCLUSIONS.

1. The administration of emulsions of the anterior lobe of the ox pituitary increases very markedly the rate of growth of the primary tumor in rats inoculated with carcinoma. The growth of small tumors is accelerated relatively more than that of large tumors.

2. This acceleration is only evidenced, however, at a certain stage in the growth of the tumor, subsequent to the twentieth day succeeding inoculation. The administrations do not enhance the tendency of the tumors to metastasize.

3. Liver emulsion does not cause an acceleration of the growth of carcinomata in rats.



## A COMPARISON OF ADULT AND INFANT TYPES OF GONOCOCCI.\*

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The group of organisms known as the gonococcus has been regarded as a heterogenous one from the point of view of the various immunological reactions. Morphologically and culturally the different strains of gonococci seem to be identical, but coincident with the use of the complement fixation test for diagnostic purposes differences in the power of various strains to bind complement were observed when monovalent antigens were used.

Torrey (1) advanced the view of the diversity of strains when studying first the agglutination and precipitin reactions in antigonococcus serum; while his absorption experiments indicated that "an antigonococcus serum may contain in addition to the specific homologous agglutinin several groups of agglutinins which act on various cultures quite independently of one another. At least three groups were found whose major or specific agglutinins are not removed by inter-absorption." Later the same author (2) confirmed this view in a study of complement fixation of the same strains. Other investigators obtained essentially similar results, although the division into three main groups was not confirmed generally. Watabiki (3), who employed the test of complement fixation in gonorrheal infections, states that "no distinct differences exist among the various strains of gonococci, but only a comparative difference." A polyvalent antigen was soon introduced for clinical diagnostic purposes. Schwartz and McNeil (4) note that "their experimental work showed that the secret of success of the serum-diagnosis of gonococcus infection in general lay in the use of a polyvalent antigen on account of the fact that the different strains of gonococci seem to differ considerably one from the other."

### SOURCES OF CULTURES.

The striking clinical distinction between gonococcal infection in the adult and in young female children suggested, in view of the facts already established by biological tests, a comparative study of gonococci isolated by culture from the two classes of cases.

Two principal groups of cases are recognized clinically. The organisms isolated from these groups were examined and compared in

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their immunological reactions of agglutination and complement fixation. Nine so called adult strains and six infant strains were studied. With one exception all were freshly isolated strains. X, Y, 1, 2, 3, 4, 5, and 6 were adult strains cultivated from cases of acute urethritis with more or less complications; A was one of the original Torrey strains and has been under cultivation for seven years. The six infant strains, BH, G, C, WE, S, and WH, were all isolated from cases of vulvovaginitis in infants and little girls under five years of age. In addition three other strains isolated from cases of ophthalmia were studied, K, L, and CM. K was isolated from a case of purulent ophthalmia in an infant two months of age; L from a girl of eighteen years who had a vaginal discharge; and CM from a boy of fifteen who had no sign or symptom of infection elsewhere and who gave no history of any exposure to contagion. Table I affords a means of easy reference.

TABLE I.  
*Source of Cultures.*

Designation.	Type of strain.	Clinical diagnosis.
X	Adult	Acute urethritis.
Y	Adult	Acute urethritis.
A (Torrey)	Adult	Acute urethritis.
1	Adult	Acute urethritis.
2	Adult	Acute urethritis.
3	Adult	Acute urethritis.
4	Adult	Acute urethritis.
5	Adult	Acute urethritis.
6	Adult	Acute urethritis.
BH	Infant	Vulvovaginitis.
G	Infant	Vulvovaginitis.
C	Infant	Vulvovaginitis.
WE	Infant	Vulvovaginitis.
S	Infant	Vulvovaginitis.
WH	Infant	Vulvovaginitis.
K	Ophthalmic strain	Ophthalmia neonatorum.
L	Ophthalmic strain	Ophthalmia; girl 18 yrs. old, with vaginitis.
CM	Ophthalmic strain	Ophthalmia; boy 15 yrs. old, with no urethritis, and no history of exposure to infection.

*Cultivation.*—As is well known, the isolation of the gonococcus from the pus in a well marked case of urethritis is comparatively easy, but the isolation of the organism from a case of vulvovaginitis



is more difficult. The easiest method of doing the latter, and one which rarely failed, was by the use of blood agar plates. The culture was taken by means of a platinum loop passed high up in the vaginal canal. If any gonococci were present they usually appeared within eighteen or twenty hours' incubation at 37° C. as the familiar tiny dew-drop colonies. Unless there was an overwhelming number of contaminating organisms in the least heavily planted plates the gonococcus colonies grew first, and in fact several hours before the other organisms appeared. If the plates are made of ascitic agar the initial growth of the contaminating organisms is more rapid than that of the gonococcus, and in eighteen hours practically every plate will be covered with them, making the selection of even probable gonococcal colonies extremely difficult. Once isolated in pure culture, the strains were grown on a stock media of ascitic veal agar.

#### EXPERIMENTAL PART.

*Immunization.*—The monovalent sera used in the immunological experiments about to be described were made by immunizing rabbits to the single adult and single infant strains. The inoculations were all intravenous ones of living twenty-four hour cultures, and were made weekly, beginning with one-twentieth or one-tenth of a culture and increasing gradually to one and one-half to two cultures. Usually eight to ten weeks sufficed to produce a serum having a good content of antibodies. The rapid method of immunization advocated by Gay (5) for the typhoid organism proved unsatisfactory when applied to the gonococcus. In each experiment detailed below serum from more than one rabbit immunized to the same strain was used and the series run in duplicate in order that any individual differences in the sera might be avoided.

*Agglutination.*—When fresh, immune rabbit serum was found to agglutinate its homologous organisms on the average in a dilution of 1:1,000, in some instances 1:1,500, and the titer of various rabbit sera did not vary appreciably whether the rabbits were immunized with a freshly isolated strain or with the same strain after it had been under cultivation for twelve months. In no instance did the agglutination titer approach the figure of 1:700,000 given by

Torrey (1). The highest titer of the Torrey A immune serum with its homologous organism was 1:666. Normal rabbit sera did not agglutinate the organism at all, or in a few instances only, in a dilution of 1:10.

Table II shows the result of the agglutination tests with the serum prepared with the adult organism X, and table III the results with the serum prepared with the infant strain BH.

TABLE II.

*Monovalent Rabbit Serum Immune to Adult Gonococcus Strain X. Agglutination Reactions Made at 37° C.*

Strain.	Control.	1:20	1:33	1:50	1:66	1:100	1:200	1:333	1:500	1:666	1:800	1:1,000	1:1,200	1:1,500	Infant serum.
X	—	±	++	++	+++	+++	+++	+++	+++	++	++	++	+	+	Adult strains.
Y	—	±	+	+	++	+++	+++	+++	++	++	++	++			
A	—	±	±	+	+	+	++	++	+	++	±	±			
1	—	+	++	++	+++	+++	+++	++	+	+	+	—			
2	—	±	+	+	+	++	++	+	±	+	—	—			
3	—	+	++	++	+	++	++	++	+	+	+	—			
4	—	±	+	++	+++	++	++	+	±	—	—	—			
5	—	±	+	++	+++	+++	+++	++	±	±	—	—			
6	—	±	+	+	++	++	+	+	—	—	—	—			
BH	—	—	—	+	+	±	—	—	—	—	—	—			Infant strains.
G	—	—	±	+	+	±	±	±	—	—	—	—			
C	—	—	±	±	+	±	+	±	—	—	—	—			
WE	—	—	±	+	+	±	±	—	—	—	—	—			
S	—	—	±	+	+	+	+	±	—	—	—	—			
WH	—	—	±	±	++	+	±	±	—	—	—	—			
K	—	±	±	+	++	++	+	+	±	—	—	—			Ophthalmic strains.
L	—	+	++	++	++	+	+	+	±	±	—	—			
CM	—	+	++	++	+	+	+	+	+	±	—	—			

It will be seen in table II that serum X (adult type) agglutinates its homologous organism and strain Y, also an adult type, equally well in dilutions of 1:1,000, and some specimens of sera agglutinated its homologous organism at 1:1,500. The average titer of other adult strains with this immune adult serum was 1:333, although strain A agglutinated at 1:666 and strains 1 and 3 at 1:800. With infant strains titrated against the adult immune serum, however, no agglutination was found in dilutions higher than 1:200, and the average was 1:66 to 1:100. The strains isolated from cases of

gonorrheal ophthalmia approach the adult type in the agglutination reaction with this adult immune serum, although above the dilution of 1:100 the reaction is not so marked as with the adult strains. The highest titer obtained with these three ophthalmic strains was 1:500 (strains L and CM).

It is interesting to compare the results given in table III with those just noted. Here the same adult strains were titered against a serum prepared with an infant type of organism, BH.

TABLE III.

*Monovalent Rabbit Serum Immune to Infant Gonococcus BH. Agglutination Reactions Made at 37° C.*

Strain.	Control.	1:20	1:33	1:50	1:66	1:100	1:200	1:333	1:500	1:666	1:800	1:1,000	1:1,200	1:1,500	Adult serum.
BH	—	±	+	++	++	+++	+++	+++	+++	+++	+++	++	+	±	Infant strains.
G	—	+	+	++	++	++	++	++	+	+	±	±			
C	—	±	±	+	+	++	++	++	+	+	+	±			
WE	—	—	+	++	++	++	++	++	++	++	+	±			
S	—	—	+	++	++	++	+	+	+	±	±	—			
WH	—	±	+	++	++	+	+	+	+	+	—	—			Adult strains.
X	—	±	±	+	+	—	—	—	—	—	—	—			
Y	—	—	±	+	+	±	—	—	—	—	—	—			
A	—	±	±	+	+	+	+	±	—	—	—	—			
1	—	+	+	+	++	+	±	±	—	—	—	—			
2	—	—	—	+	+	±	±	—	—	—	—	—			
3	—	+	+	++	++	++	+	±	—	—	—	—			
4	—	—	±	+	+	+	+	—	—	—	—	—			
5	—	—	—	+	++	+	+	—	—	—	—	—			
6	—	—	+	+	+	+	±	—	—	—	—	—			
K	—	+	+	++	+	+	±	±	—	—	—	—			Ophthalmic strains.
L	—	+	+	+	++	+	±	—	—	—	—	—			
CM	—	+	+	++	+	±	±	—	—	—	—	—			

The titer of this serum with its homologous organism was high, 1:1,000, and with some specimens of sera 1:1,200, but the titer of two other infant strains, C and WE, was also high, 1:800. The average titer of this infant immune serum with infant strains was 1:500. On the other hand, when adult strains were used with this infant immune serum rather striking differences were again brought out. In no case did agglutination occur in dilution of the serum above 1:200, and the average was 1:100. Strains 1, 3, and 5 were the only ones showing a double plus reaction, and this occurred in

dilutions of 1:66 and 1:100. The three ophthalmic strains when titrated against this infant immune serum did not agglutinate in dilutions above 1:100, thus conforming again to the adult type.

As will be seen in tables II and III, a pre-zone of agglutination was present quite regularly. It was seen in all infant strains tested with adult immune serum; strains 1 and 3 never exhibited it when tested with either adult or infant immune sera. The ophthalmic strains practically never showed it; there was a plus-minus reaction of strain K with the lowest dilution of the adult immune serum. When the homologous strains were tested with their own sera there was a plus-minus reaction in the lowest dilution, suggesting a pre-zone when compared with the plus reaction in the succeeding higher dilution, yet not as definite a one as with other strains or when the two strains X (adult) and BH (infant) were crossed with the opposite serum.

TABLE IV.

*Monovalent Rabbit Serum Immune to Adult Strain Y. Agglutination Reactions Made at 37° C.*

Strain.	Control.	1:20	1:33	1:50	1:66	1:100	1:200	1:333	1:500	1:666	1:800	1:1,000	Adult serum
Y	—	++	+++	+++	+++	+++	+++	+++	+++	++	++	++	Adult strains.
X	—	—	+++	+++	+++	+++	+++	++	++	++	++	++	
1	—	+	+	+	+	+	+	+	+	—	—	—	
4	—	++	+	+	++	+++	++	+	+	—	—	—	
BH	—	—	+	+	++	+	—	—	—	—	—	—	Infant strains.
G	—	—	—	+	+	++	—	—	—	—	—	—	
C	—	—	—	+	+	+	++	—	—	—	—	—	
WE	—	—	++	+	+	+	++	++	—	—	—	—	

*Monovalent Rabbit Serum Immune to Infant Strain C.*

Strain.	Control.	1:20	1:33	1:50	1:66	1:100	1:200	1:333	1:500	1:666	1:800	1:1,000	Infant serum
C	—	++	+++	+++	+++	+++	+++	+++	+++	+++	+++	++	Infant strains.
BH	—	++	+++	+++	+++	+++	++	+++	++	+	+	+	
G	—	—	+	+	+	++	+	+	+	+	+	+	
WE	—	—	+	+	++	+++	++	++	++	+	—	—	
Y	—	—	+	+	+	+	++	—	—	—	—	—	Adult strains.
X	—	++	+++	+++	+	++	++	—	—	—	—	—	
1	—	+	+	+	+	+	+	—	—	—	—	—	
4	—	—	++	++	+	++	++	—	—	—	—	—	

In addition to the experiments given in the tables others were made of a similar nature, but with sera immune to other adult and other infant strains; *e. g.*, Y and A, C and WE. The results were practically the same as with X and BH immune sera. Table IV gives some of the agglutination reactions with serum prepared with Y (adult) and serum prepared with C (infant) strains.

*Complement Fixation.*—Antigens were made according to several different methods, but the one finally adopted was that given by Park and Williams (6). We found in making these monovalent antigens that it made no difference whether the final heating at 80° C. for one hour was done before or after filtering, or whether the autolysis at 56° C. lasted for one hour or two. Moreover, in the few instances tried there was no difference noted in the same antigen heated at 80° C. for one hour or at 100° C. for twenty minutes. The antigen is used the same day as prepared, if possible, for in some instances it becomes anticomplementary over night. When an antigen is anticomplementary in dilutions commonly used or becomes so in such dilutions as to prevent its being used, refiltering it or reheating it will not remove its anticomplementary character.

The sheep-rabbit hemolytic system was used throughout these experiments, as it was found that the serum of both normal rabbits and those immunized with the gonococcus contained a stronger natural hemolytic amboceptor for hen than for the sheep red corpuscles.

Before performing each experiment a careful titration of the hemolytic system was done, with varying amounts of amboceptor and complement, and for the final test two units of amboceptor and two of complement were used. Moreover, an equally careful titration of the antigen was made and in each case one-half the amount of antigen that was entirely free from anticomplementary action was used. Harrison (7) lays especial stress on the importance of these two initial titrations and our experience confirms his. The tests were all made in the water bath at 37° C.; antigen, immune serum, and complement were incubated for forty-five minutes, then the hemolytic amboceptor and sheep red corpuscles were added, and the total mixture was incubated for one hour. Table V shows the results of antigens made from adult type organisms tested with both adult and infant type immune sera.

TABLE V.

*Monovalent Adult Type Antigens Tested for Complement Deviation.*

Strain, Dilution of antigen.	Dilution of immune sera.		Serum X (adult).	Serum BH (infant).	Complement.
X (adult) 0.2 c.c. in each tube	0.4	c.c.	No hemolysis	No hemolysis	0.01 c.c. in each tube.
	0.3	c.c.	No hemolysis	Slight hemolysis	
	0.2	c.c.	No hemolysis	Complete hemolysis	
	0.1	c.c.	No hemolysis	Complete hemolysis	
	0.07	c.c.	No hemolysis	Complete hemolysis	
	0.05	c.c.	No hemolysis	Complete hemolysis	
	0.03	c.c.	No hemolysis	Complete hemolysis	
	0.01	c.c.	Moderate hemolysis	Complete hemolysis	
	0.007	c.c.	Complete hemolysis	Complete hemolysis	
Y (adult) 0.2 c.c. in each tube	0.4	c.c.	No hemolysis	No hemolysis	0.01 c.c. in each tube.
	0.3	c.c.	No hemolysis	Complete hemolysis	
	0.2	c.c.	No hemolysis	Complete hemolysis	
	0.1	c.c.	No hemolysis	Complete hemolysis	
	0.07	c.c.	No hemolysis	Complete hemolysis	
	0.05	c.c.	No hemolysis	Complete hemolysis	
	0.03	c.c.	Complete hemolysis	Complete hemolysis	
	0.01	c.c.	Complete hemolysis	Complete hemolysis	
	0.007	c.c.	Complete hemolysis	Complete hemolysis	
A (adult) 0.3 c.c. in each tube	0.4	c.c.	No hemolysis	No hemolysis	0.01 c.c. in each tube.
	0.3	c.c.	No hemolysis	Complete hemolysis	
	0.2	c.c.	Slight hemolysis	Complete hemolysis	
	0.1	c.c.	Slight hemolysis	Complete hemolysis	
	0.07	c.c.	Complete hemolysis	Complete hemolysis	
	0.05	c.c.	Complete hemolysis	Complete hemolysis	
	0.03	c.c.	Complete hemolysis	Complete hemolysis	
	0.01	c.c.	Complete hemolysis	Complete hemolysis	
	0.007	c.c.	Complete hemolysis	Complete hemolysis	
1 (adult) 0.2 c.c. in each tube	0.4	c.c.	No hemolysis	No hemolysis	0.01 c.c. in each tube.
	0.3	c.c.	No hemolysis	No hemolysis	
	0.2	c.c.	No hemolysis	No hemolysis	
	0.1	c.c.	No hemolysis	Complete hemolysis	
	0.07	c.c.	Slight hemolysis	Complete hemolysis	
	0.05	c.c.	Complete hemolysis	Complete hemolysis	
	0.03	c.c.	Complete hemolysis	Complete hemolysis	
	0.01	c.c.	Complete hemolysis	Complete hemolysis	
	0.007	c.c.	Complete hemolysis	Complete hemolysis	
2 (adult) 0.2 c.c. in each tube	0.4	c.c.	No hemolysis	No hemolysis	0.01 c.c. in each tube.
	0.3	c.c.	No hemolysis	No hemolysis	
	0.2	c.c.	No hemolysis	No hemolysis	
	0.1	c.c.	Slight hemolysis	Complete hemolysis	
	0.07	c.c.	Complete hemolysis	Complete hemolysis	
	0.05	c.c.	Complete hemolysis	Complete hemolysis	
	0.03	c.c.	Complete hemolysis	Complete hemolysis	
	0.01	c.c.	Complete hemolysis	Complete hemolysis	
	0.007	c.c.	Complete hemolysis	Complete hemolysis	

TABLE V.—*Concluded.*

Strain. Dilution of antigen.	Dilution of immune sera.		Serum X (adult).	Serum BH (infant).	Complement.
3 (adult) 0.2 c.c. in each tube	0.4	c.c.	No hemolysis	No hemolysis	0.01 c.c. in each tube.
	0.3	c.c.	No hemolysis	No hemolysis	
	0.2	c.c.	No hemolysis	Moderate hemolysis	
	0.1	c.c.	No hemolysis	Moderate hemolysis	
	0.07	c.c.	No hemolysis	Complete hemolysis	
	0.05	c.c.	Slight hemolysis	Complete hemolysis	
	0.03	c.c.	Complete hemolysis	Complete hemolysis	
	0.01	c.c.	Complete hemolysis	Complete hemolysis	
	0.007	c.c.	Complete hemolysis	Complete hemolysis	
4 (adult) 0.2 c.c. in each tube	0.4	c.c.	No hemolysis	No hemolysis	0.01 c.c. in each tube.
	0.3	c.c.	No hemolysis	No hemolysis	
	0.2	c.c.	No hemolysis	Slight hemolysis	
	0.1	c.c.	No hemolysis	Complete hemolysis	
	0.07	c.c.	No hemolysis	Complete hemolysis	
	0.05	c.c.	No hemolysis	Complete hemolysis	
	0.03	c.c.	Complete hemolysis	Complete hemolysis	
	0.01	c.c.	Complete hemolysis	Complete hemolysis	
	0.007	c.c.	Complete hemolysis	Complete hemolysis	
5 (adult) 0.2 c.c. in each tube	0.4	c.c.	No hemolysis	No hemolysis	0.01 c.c. in each tube.
	0.3	c.c.	No hemolysis	No hemolysis	
	0.2	c.c.	No hemolysis	Complete hemolysis	
	0.1	c.c.	No hemolysis	Complete hemolysis	
	0.07	c.c.	No hemolysis	Complete hemolysis	
	0.05	c.c.	Complete hemolysis	Complete hemolysis	
	0.03	c.c.	Complete hemolysis	Complete hemolysis	
	0.01	c.c.	Complete hemolysis	Complete hemolysis	
	0.007	c.c.	Complete hemolysis	Complete hemolysis	

Antigens made from strain 6 were repeatedly anticomplementary in as small amounts as 0.05 c.c.

When monovalent antigens made from the so called adult strains of gonococci were titrated against a monovalent immune serum prepared with an adult strain, complement was bound in dilutions which caused no deviation of the complement when tested with a so called infant type of immune serum. Sera X (adult type) and BH (infant type) were used. Antigens X (adult) and Y (adult) show practically the same results in this test; complement is bound with 0.05 of a cubic centimeter of adult immune serum, but there is no binding whatever with 0.2 of a cubic centimeter of infant immune serum.

This result happens to coincide with the agglutination tests made with these strains and the same adult serum, but it by no means follows that because of a high agglutination titer complement will

be bound with high dilutions of the serum. It has been shown repeatedly that a high content of a serum of one group or set of antibodies does not necessarily imply a high content of others. This is illustrated with strains 1 (adult) and 4 (adult) and immune serum X (adult). With antigen 1 there is only partial binding of complement with 0.07 of a cubic centimeter, while with antigen 4 there was complete binding with 0.05 of a cubic centimeter. Yet the agglutination tests made with these strains and serum show that there was a partial agglutination of strain 1 at a serum dilution of 1:666, while with strain 4 agglutination occurred at a dilution of 1:333 (table II). In every instance complement was bound in the lowest dilutions of the infant immune serum as well as the adult, with the adult antigens, but never with smaller amounts than 0.3 of a cubic centimeter of the infant serum. On the other hand, with these adult antigens complement was always bound with 0.3 of a cubic centimeter of adult immune serum, in every case with 0.1 of a cubic centimeter or less, and in one experiment with 0.03 of a cubic centimeter (antigen X, serum X). Antigens made from strain 6 according to the method used were repeatedly anticomplementary in as high a dilution as 0.05 of a cubic centimeter. An explanation of this is not apparent, but it may be possible to make a usable antigen from this strain by another method; *i. e.*, the freezing and thawing method. Strain 6 was agglutinated with an adult immune serum in a dilution of 1:333 (table II) and with an infant immune serum at 1:100 (table III). Hence it is classed with the adult type of organism. This is borne out by the history of the case which shows that it was isolated from an adult urethritis (table I).

When the infant strain antigens were titrated against the same monovalent infant and adult immune sera (BH and X), similar differences were observed, as shown in table VI.

Complement was bound with 0.03 of a cubic centimeter of BH serum with BH antigen, but there was no binding whatever with this amount of X (adult) serum. In every case complement was bound with 0.1 of a cubic centimeter of infant serum and usually with 0.05 of a cubic centimeter, when these infant strain antigens were used; but there was no binding whatever with this amount of adult immune serum. With infant type antigens complement was bound



TABLE VI.

*Monovalent Infant Type Antigens Tested for Complement Deviation.*

Strain. Dilution of antigen.	Dilution of immune sera.	Serum BH (infant).	Serum X (adult).	Complement.
BH (infant) 0.3 c.c. in each tube	0.4 c.c.	No hemolysis	No hemolysis	0.01 c.c. in each tube.
	0.3 c.c.	No hemolysis	No hemolysis	
	0.2 c.c.	No hemolysis.	Slight hemolysis	
	0.1 c.c.	No hemolysis	Complete hemolysis	
	0.07 c.c.	No hemolysis	Complete hemolysis	
	0.05 c.c.	No hemolysis	Complete hemolysis	
	0.03 c.c.	No hemolysis	Complete hemolysis	
	0.01 c.c.	Complete hemolysis	Complete hemolysis	
	0.007 c.c.	Complete hemolysis	Complete hemolysis	
G (infant) 0.2 c.c. in each tube	0.4 c.c.	No hemolysis	No hemolysis	0.01 c.c. in each tube.
	0.3 c.c.	No hemolysis	No hemolysis	
	0.2 c.c.	No hemolysis	No hemolysis	
	0.1 c.c.	No hemolysis	Complete hemolysis	
	0.07 c.c.	Moderate hemolysis	Complete hemolysis	
	0.05 c.c.	Complete hemolysis	Complete hemolysis	
	0.03 c.c.	Complete hemolysis	Complete hemolysis	
	0.01 c.c.	Complete hemolysis	Complete hemolysis	
	0.007 c.c.	Complete hemolysis	Complete hemolysis	
C (infant) 0.3 c.c. in each tube	0.4 c.c.	No hemolysis	No hemolysis	0.01 c.c. in each tube.
	0.3 c.c.	No hemolysis	No hemolysis	
	0.2 c.c.	No hemolysis	No hemolysis	
	0.1 c.c.	No hemolysis	Complete hemolysis	
	0.07 c.c.	No hemolysis	Complete hemolysis	
	0.05 c.c.	No hemolysis	Complete hemolysis	
	0.03 c.c.	Slight hemolysis	Complete hemolysis	
	0.01 c.c.	Complete hemolysis	Complete hemolysis	
	0.007 c.c.	Complete hemolysis	Complete hemolysis	
WE (infant) 0.3 c.c. in each tube	0.4 c.c.	No hemolysis	No hemolysis	0.01 c.c. in each tube.
	0.3 c.c.	No hemolysis	No hemolysis	
	0.2 c.c.	No hemolysis	No hemolysis	
	0.1 c.c.	No hemolysis	Moderate hemolysis	
	0.07 c.c.	No hemolysis	Complete hemolysis	
	0.05 c.c.	Almost complete hemolysis	Complete hemolysis	
	0.03 c.c.	Complete hemolysis	Complete hemolysis	
	0.01 c.c.	Complete hemolysis	Complete hemolysis	
	0.007 c.c.	Complete hemolysis	Complete hemolysis	
S (infant) 0.2 c.c. in each tube	0.4 c.c.	No hemolysis	No hemolysis	0.01 c.c. in each tube.
	0.3 c.c.	No hemolysis	No hemolysis	
	0.2 c.c.	No hemolysis	Complete hemolysis	
	0.1 c.c.	Slight hemolysis	Complete hemolysis	
	0.07 c.c.	Moderate hemolysis	Complete hemolysis	
	0.05 c.c.	Complete hemolysis	Complete hemolysis	
	0.03 c.c.	Complete hemolysis	Complete hemolysis	
	0.01 c.c.	Complete hemolysis	Complete hemolysis	
	0.007 c.c.	Complete hemolysis	Complete hemolysis	

TABLE VI.—*Concluded.*

Strain, Dilution of antigen.	Dilution of immune sera.	Serum BH (infant).	Serum X (adult).	Complement.
WH (infant) 0.2 c.c. in each tube	0.4 c.c.	No hemolysis	No hemolysis	0.01 c.c. in each tube.
	0.3 c.c.	No hemolysis	No hemolysis	
	0.2 c.c.	No hemolysis	No hemolysis	
	0.1 c.c.	No hemolysis	Moderate hemolysis	
	0.07 c.c.	No hemolysis	Complete hemolysis	
	0.05 c.c.	No hemolysis	Complete hemolysis	
	0.03 c.c.	Complete hemolysis	Complete hemolysis	
	0.01 c.c.	Complete hemolysis	Complete hemolysis	
	0.007 c.c.	Complete hemolysis	Complete hemolysis	

with somewhat smaller amounts of adult immune serum (averaging 0.2 of a cubic centimeter) than was the case with adult type antigens and infant immune serum (averaging 0.3 of a cubic centimeter). Again it is seen that a high agglutination titer does not imply necessarily a deviation of complement at a high dilution of the serum (strain G).

Antigens made from the three organisms isolated from ophthalmic cases when titrated against monovalent adult serum deviated complement in higher dilutions of this serum than when titrated against infant immune serum (table VII). This difference was well marked with strain CM, but less striking with strains K and L.

It should be emphasized again that in order to determine any degree of specificity whatever of the various strains of gonococci, the smallest amount of complement possible was used (two units) with the largest amount of antigen and immune serum (half the amount that was not anticomplementary). The test so made is an extremely delicate one. It is hardly necessary to add that such quantities would not be practical for the ordinary clinical procedure.

#### DISCUSSION.

From the agglutination and complement fixation tests just described it is evident that within the large group of organisms known as the gonococcus two more or less distinct types may be differentiated. These types correspond to the clinical source of the strains, whether from an infection of the acute urethritis type in the adult or from the vulvovaginitis of infancy and early childhood. The difference between these types of gonorrheal infection is well recog-

TABLE VII.

*Monovalent Ophthalmic Gonococcus Antigens Tested for Complement Deviation.*

Strain. Dilution of antigen.	Dilution of immune sera.	Serum X (adult).	Serum BH (infant).	Complement.
K (ophthalmic) 0.2 c.c. in each tube	0.4 c.c.	No hemolysis	No hemolysis	0.01 c.c. in each tube.
	0.3 c.c.	No hemolysis	No hemolysis	
	0.2 c.c.	No hemolysis	Complete hemolysis	
	0.1 c.c.	Moderate hemolysis	Complete hemolysis	
	0.07 c.c.	Complete hemolysis	Complete hemolysis	
	0.05 c.c.	Complete hemolysis	Complete hemolysis	
	0.03 c.c.	Complete hemolysis	Complete hemolysis	
	0.02 c.c.	Complete hemolysis	Complete hemolysis	
	0.01 c.c.	Complete hemolysis	Complete hemolysis	
	0.007 c.c.	Complete hemolysis	Complete hemolysis	
L (ophthalmic) 0.2 c.c. in each tube	0.4 c.c.	No hemolysis	No hemolysis	0.01 c.c. in each tube.
	0.3 c.c.	No hemolysis	No hemolysis	
	0.2 c.c.	No hemolysis	No hemolysis	
	0.1 c.c.	No hemolysis	No hemolysis	
	0.07 c.c.	No hemolysis	Complete hemolysis	
	0.05 c.c.	Complete hemolysis	Complete hemolysis	
	0.03 c.c.	Complete hemolysis	Complete hemolysis	
	0.01 c.c.	Complete hemolysis	Complete hemolysis	
	0.007 c.c.	Complete hemolysis	Complete hemolysis	
CM (ophthalmic) 0.2 c.c. in each tube	0.4 c.c.	No hemolysis	No hemolysis	0.01 c.c. in each tube.
	0.3 c.c.	No hemolysis	No hemolysis	
	0.2 c.c.	No hemolysis	No hemolysis	
	0.1 c.c.	No hemolysis	No hemolysis	
	0.07 c.c.	No hemolysis	Complete hemolysis	
	0.05 c.c.	No hemolysis	Complete hemolysis	
	0.03 c.c.	Complete hemolysis	Complete hemolysis	
	0.01 c.c.	Complete hemolysis	Complete hemolysis	
	0.007 c.c.	Complete hemolysis	Complete hemolysis	

nized clinically and the immunological tests herewith presented would seem to offer an experimental indication that at least a part of the difference is due to an inherent difference in the two types of organisms causing the infections. It is highly probable that gradations between the two types exist; that the gonococcus may be more or less of a labile group within itself; and that if a large enough number of strains were tested by their immunological reactions certain of these would be found partaking of the characteristics of both types. The conclusion is not to be drawn that the adult and infant types of gonococci form two distinct clear-cut groups separate one from the other, but that certain distinctions exist between them, relative differences which can be brought out by the immunological reactions of agglutination and complement fixation with specific

immune sera. But the distinction between the two types is not an absolute one.

We may add at this point that in no instance did any agglutination of either type of gonococcus occur with sera immune to *Diplococcus intracellularis* or to *Micrococcus catarrhalis*. Moreover, there was no binding of complement with meningococcus antigen and gonococcus immune serum, and none with gonococcus antigens (adult or infant type) with either meningococcus or *catarrhalis* immune serum.

Schwartz and McNeil (4) state in their paper that in the ten cases examined of vulvovaginitis in children under five years of age, the complement fixation test of the patient's blood was negative, although in all the gonococcus was demonstrated in smears and by culture. They thought that the negative results were due to the fact that there was no involvement of the cervix, only the vulva, urethra, and vagina being affected, and that unless the cervix was involved, a positive complement fixation test could not be obtained. In the light of our experiments we feel that the reason the tests were negative may have been because a suitable specific antigen was not used. A polyvalent antigen, it is true, was used, but one made from adult strains only. Therefore, in making an antigen for the clinical complement fixation test for gonococcal infection it would be well to employ strains obtained from infants as well as from adults.

#### CONCLUSIONS.

1. Two principal types of gonococci may be recognized by suitable immunological tests; *e. g.*, agglutination and complement fixation. These two types correspond to the adult and infant types of infection with the gonococcus, seen clinically.
2. On the basis of the immunological reactions of agglutination and complement fixation the strains of gonococci isolated from three cases of ophthalmia are classed with the adult type.
3. A polyvalent antigen for the serological diagnosis of a gonococcus infection should represent strains of the infant type of organism in order that both types of infection, infant as well as adult, may be recognized.

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## STUDIES UPON EXPERIMENTAL PNEUMONIA IN RABBITS.

### VII. THE PRODUCTION OF LOBAR PNEUMONIA.\*

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In a previous paper the production of pneumonia in rabbits by the Meltzer method of intratracheal inoculation was reported (1). The results by this method have not been constant in the hands of all observers (2). An investigation of the details of the method was undertaken with the hope of determining the causes of this inconsistency.

During the past two years a series of animals has been inoculated intratracheally with pneumococci, but not all the animals developed lobar pneumonia. The only factors which may have varied were the force with which the culture was injected into the respiratory tract and the distance to which the catheter was inserted.

A series of experiments was done to determine the importance of these variables. The force with which the culture was injected was readily controlled. In all cases a Record syringe was used containing five to six cubic centimeters of culture fluid and about fifteen cubic centimeters of air. In the animals in which it was desired to inject the culture with great force, the piston of the syringe was pushed in as rapidly as possible. In those in which it was desired to inject with little force, the piston was pushed in very slowly.

In half the animals the catheter was inserted deeply into a bronchus. In the remainder it was pushed into the trachea just a short way beyond the larynx. The results are recorded in table I.

*Discussion of Table I.*—From table I it appears that, in order to produce lobar pneumonia in rabbits, it is necessary to introduce the organisms into the deeper portions of the respiratory tract, and probably into the air sacs themselves. This can be accomplished

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TABLE I.

Animal No.	Position of catheter.		Force of injection.		Lesions.	
	Deep in bronchus.	Just beyond larynx.	Great.	Slight.	Lobar pneumonia.	Bronchopneumonia.
231	.....	+	.....	+	.....	.....
232	.....	+	.....	+	.....	+
254	.....	+	.....	+	.....	.....
256	.....	+	.....	+	.....	+
255	.....	+	.....	+	.....	.....
235	.....	+	+	.....	.....	.....
236	.....	+	+	.....	.....	.....
251	.....	+	+	.....	.....	.....
252	.....	+	+	.....	.....	.....
253	.....	+	+	.....	.....	+
229	+	.....	.....	+	.....	+
230	+	.....	.....	+	+	.....
248	+	.....	.....	+	+	.....
249	+	.....	.....	+	+	.....
250	+	.....	.....	+	+	.....
233	+	.....	+	.....	+	.....
234	+	.....	+	.....	+	.....
254A	+	.....	+	.....	+	.....
254B	+	.....	+	.....	+	.....
246	+	.....	+	.....	+	.....

*Summary of Table I.*—Table I may be summarized as follows: In the first five animals the culture was gently injected into the trachea just beyond the larynx. At the end of three days the animals seemed normal, but were killed to see what changes were present in the respiratory tract. None of them showed lobar pneumonia, and only two showed a minute patch of bronchopneumonia.

In the second five animals the culture was forcibly injected into the trachea just beyond the larynx. One of these animals died at the end of forty-one hours and showed a small area of consolidation in the right middle lobe. The other four animals were in good condition at the end of three days, when they were killed. In none of them was there any evidence of either lobar or lobular pneumonia.

In the third five animals the culture was gently injected as far down one bronchus as the catheter could be inserted. Two of these animals died in forty-two and forty-five hours, respectively, and both showed lobar consolidation. The other three animals were killed at the end of three days. Two of these had lobar pneumonia while the third had patchy bronchopneumonia.

In the last five animals the culture was forcibly injected as far down one bronchus as the catheter could be inserted. All these animals died in from twenty to forty-six hours. They all showed lobar consolidation.

only by the insertion of the catheter as deeply into the bronchi as possible. Even under these conditions, unless the culture fluid is injected with considerable force, lobar pneumonia will not necessarily result. If the culture is introduced into the upper air pas-

sages with the catheter just beyond the larynx, the lungs are rarely affected.

This suggests that there may be some protective mechanism in the upper respiratory tract whereby the organisms are prevented from reaching the alveoli; and in man, also, the development of pneumonia may be associated with the imperfect functioning of such a mechanism.

During recent years there has been some evidence that pneumonia is a contagion; but while there can be no doubt that this is true at times, it is not apparent in the vast majority of cases. It is generally believed that pneumonia does not develop from infection from without, but that the organisms dwelling upon the mucous membrane of the upper respiratory tract are the cause of the disease. There is no question that if these organisms of the upper respiratory tract reach the alveoli of the lungs in sufficient numbers they will produce pneumonia, just as many other organisms, regardless of their virulence, when experimentally introduced into the alveoli, will produce pneumonia (3).

We have no conclusive evidence in support of the theory that the organisms normally present in the mouth and upper respiratory passages are the cause of pneumonia in man, but, granting this, the terms lowered resistance and predisposition to this disease must mean a disturbance of the protective mechanism of the upper respiratory tract.

This is clearly demonstrated in the experiments noted above, in which animals receiving considerable numbers of pneumococci in the trachea, just beyond the larynx, get rid of them without suffering appreciable involvement of the lungs; whereas those animals in which the same number of similar organisms has been introduced into the air sacs invariably develop lobar pneumonia.

A series of experiments was undertaken to determine the importance of such secondary factors in the etiology of pneumonia, as alcohol, cold, inhalation of ether and bromine, and the control of the vagus upon the upper air passages.

#### EFFECT OF ALCOHOL AND COLD.

Seven animals were given from 20 to 40 c.c. of 30 per cent. alcohol daily from one to nine days by stomach tube, according to the method of Frieden-



wald (4). Each day, following the administration of alcohol, the animals were placed in a cool chamber at about 4° C. for a few hours. One of the animals so treated for three days was killed and showed patchy congestion in various lobes. Four others died after they had been given alcohol and exposed to cold. Two of these had intense laryngitis, tracheitis, and bronchitis, and the fourth showed scattered patches of bronchopneumonia. In each of the two remaining animals 1.5 c.c. of a twenty-four hour culture of pneumococci were gently injected into the trachea just beyond the larynx, after the animals had been given alcohol and exposed to cold on nine consecutive days. Death followed in two and three days, respectively. One animal showed uniform congestion of all lobes with intense laryngitis, tracheitis, and bronchitis. The other showed a patch of bronchopneumonia involving about one-twelfth of the left lobe, together with injection of the larynx, trachea, and bronchi.

#### EFFECT OF ETHER.

Five animals were anesthetized with ether and then 5 c.c. of a twenty-four hour culture of pneumococci in pig serum broth were gently injected into the trachea of each with the catheter inserted just beyond the larynx. The ether anesthesia was continued. Two of the animals died after one half hour's inhalation, and each showed moderate congestion of all lobes. In the remaining three the anesthesia was continued for one hour. At the end of three days the animals were killed. One showed a purulent and fibrinopurulent pleurisy on the right side. The two remaining animals showed patchy consolidation of the left lower lobe.

#### EFFECT OF BROMINE INHALATION.

Eight animals were subjected to bromine fumes one hour daily for three to ten days. After each exposure the mucous membrane of the nose and mouth was found injected, slightly swollen, and covered with a thin, watery exudate.

In three of these animals 5 c.c. of a culture of pneumococci in pig serum broth were gently injected into the trachea beyond the larynx just before the last bromine exposure. At the end of forty-eight hours the animals were killed. Two showed injection of the larynx, trachea, and bronchi. The third showed acute laryngitis, tracheitis, and bronchitis, and a few small patches of bronchopneumonia in both lungs.

In three of the remaining animals 5 c.c. of a culture of pneumococci in pig serum broth were gently injected into the trachea beyond the larynx a few minutes after the last bromine exposure. These animals were sacrificed at the end of forty-eight hours. One of them showed injection of the trachea; the others showed injection of the larynx and trachea and a few small patches of consolidation in two lobes.

The two remaining animals in the series were given no pneumococci, but were killed forty-eight hours after the last bromine exposure. One showed a diffuse injection of the larynx, trachea, and bronchi; the other showed marked injection of the larynx, trachea, and large and small bronchi, with extensive hemorrhages of the smaller bronchi extending into the adjoining alveoli.

The results of the experiments to determine the influence of alcohol and cold and the inhalation of ether and bromine in the etiology of lobar pneumonia are not conclusive. These factors seem to predispose toward the development of a bronchitis and even bronchopneumonia, both in animals receiving organisms into the upper air passage, and in animals not so treated.

#### EFFECT OF CUTTING ONE VAGUS NERVE.

In this experiment seven animals were used. In four about one inch of the right vagus nerve (midcervical portion) was resected, and two days later 5 c.c. of a twenty hour culture of pneumococci were gently injected into the trachea just beyond the larynx. At the end of three days the animals were sacrificed. One showed no pulmonary change whatever; one showed a purulent and fibrinopurulent pleurisy and mediastinitis, but no involvement of the lungs; the third had a small patch of consolidation in the right lower lobe; and the fourth showed a patch of consolidation, lobar in type, affecting about one-sixth of the left lower lobe, congestion of the left upper lobe, and bilateral purulent and fibrinopurulent pleurisy.

The fifth animal received 4 c.c. of a twenty-four hour culture of pneumococci in the trachea beyond the larynx eleven and one-half weeks after a right-sided vagotomy. The rabbit was killed seven days later and showed purulent bronchitis, atelectasis of both upper lobes, and extensive fibrinopurulent peritonitis.

The two remaining animals died in three and twenty-two days, respectively, after simple right-sided vagotomy. The first showed early lobar consolidation of the entire right, middle, and left upper lobes, and one-half of the left lower lobe. The other animal showed lobar consolidation of one-half of the right lower lobe and fibrinopurulent pleurisy, mediastinitis, and pericarditis.

*B. coli* was obtained in pure culture from the consolidated lung of the animal that died three days after vagotomy. In this connection the results of cultures taken from lungs of rabbits developing pneumonia spontaneously are of interest. The disease is not of rare occurrence. A number of the stock animals as well as several dying in the country and brought to the laboratory for investigation showed this lesion. The associated organism was usually one of the intestinal group (*B. coli*, *B. lactis aerogenes*), although in one instance a pneumococcus was obtained in pure culture.

#### EFFECT OF CUTTING BOTH VAGI.

Six rabbits were used for this experiment. In five both vagi were resected at the same time. In the sixth the left vagus was cut seven days after a right-sided vagotomy. Although no organisms were injected into the respiratory tract the animals died five to forty-eight hours after both vagi were sectioned. The two animals dying in five hours showed patchy congestion of all lobes. In one there was, in addition, considerable tracheitis and bronchitis. Of the two that died in ten hours, one had patches of congestion in all lobes and both showed bronchitis. The fifth died in sixteen hours and showed intense laryngitis, tra-

cheitis, and bronchitis, bilateral purulent and fibrinopurulent pleurisy, and extensive congestion and early consolidation of all lobes, especially the right lower. The last animal showed pseudolobar consolidation of the entire left upper lobe (innumerable adjoining areas of grayish red consolidation) and congestion and edema of all other lobes. This animal also had marked laryngitis, tracheitis, and bronchitis, and bilateral fibrinopurulent pleurisy. In all but the last animal the esophagus was found engorged with partly digested food and, in one instance, a considerable amount of this material was found in the trachea.

These results are in accord with the findings of previous investigators. There is an abundance of literature upon the relation of the vagus nerve to pneumonia, with various theories of explanation. The controversy of Traube (5) and Schiff (6) (1848-1871) has been followed by the observations and conclusions of many others (Vulpian (7), Piccinino (8), Bettini (9)).

In brief it may be stated that as long ago as 1740 Valsalva (10) noted the occurrence of pneumonia in laboratory animals following double vagotomy.

In 1846 Traube called attention to paralysis of the esophagus as well as the larynx in these animals. Contrary to Schiff, who ascribed the affection to a vasomotor disturbance following the section, Traube attributed the disease to the aspiration into the respiratory tract of saliva and food particles. In support of this theory his demonstration of the ability to produce pneumonia by the injection of saliva and food particles in otherwise untreated animals and the possibility of averting the disease by completely separating the digestive from the respiratory tract is significant.

The above results in animals with both vagi cut reveal the importance of the control of these nerves upon the respiratory tract. When they are both out of function the protective mechanism of the upper respiratory tract is no longer operative, and organisms are allowed to penetrate to the alveoli and there set up an inflammatory process.

#### CONCLUSIONS.

1. The production of lobar pneumonia in rabbits is dependent upon the introduction of organisms into the alveoli themselves.

2. In order to accomplish this the catheter through which they are injected must be inserted as deeply into a bronchus as possible and the culture fluid injected with considerable force.

3. Large numbers of organisms injected into the trachea just beyond the larynx set up no great changes in the lungs, even though the injection be forcible. This fact suggests the presence of a protective mechanism in the upper air passages, which, under normal conditions, prevents the penetration of organisms into the lungs.

4. If animals be subjected to cold, alcohol, and the inhalation of

irritating gases, the so called secondary factors in the etiology of lobar pneumonia in man, then the injection of pneumococci into the trachea causes inflammatory changes of the upper respiratory tract and occasionally pneumonia.

5. The vagi prevent foreign material in the pharynx and upper respiratory tract from reaching the lungs. Section of one vagus may be followed by pneumonia, while section of both invariably leads to this result.

6. It is possible that the secondary factors mentioned above owe their action to their influence upon the vagus control of the upper respiratory tract.

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## STUDIES UPON EXPERIMENTAL PNEUMONIA IN RABBITS.

### VIII. INTRA VITAM STAINING IN EXPERIMENTAL PNEUMONIA, AND THE CIRCULATION IN THE PNEUMONIC LUNG.\*

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It has been shown (1) that when pneumonia is produced in a vitally stained animal, the consolidated area is intensely stained, while the remainder of the lung remains very faintly colored. The colorization of the pneumonic area is dependent upon the localization of the dye in the fibrin of the exudate. The nuclei of some of the leucocytes are also stained.

On the basis of these preliminary experiments it was decided to test more carefully the affinity of the dye for the various elements of the exudate at intervals in the progress of pneumonia. In the course of this study phenomena presented themselves which led to a more detailed examination of the circulation in the pneumonic lung.

#### INTRA VITAM STAINING IN EXPERIMENTAL PNEUMONIA.

The stain used in the experiments was trypan blue. The method of injection was the same as that described in a previous paper (2).

As is well known from the studies of Bouffard (3), Goldmann (4), and others, the tissues of the lung have very little affinity for the dye, and in normal, vitally stained animals only a few of the cells of the interstitial tissue contain blue granules in their protoplasm. Consequently, the lung appears pale pink in contrast to the liver, for example, which is intensely stained.

Schulemann (5) and others have pointed out that the cells of the circulating blood do not absorb the dye, and the only cells that may appear in the blood containing it are huge macrophages similar to those found in the serous cavities of the body. These cells are not found in the pneumonic exudate which is composed of blood cells, chiefly polymorphonuclear leucocytes.

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It has been shown in the unpublished experiments of Evans and Winternitz that the nucleus of a living cell never stains vitally, but that as soon as a cell is injured its nucleus absorbs the stain. In the hands of other observers, Evans and MacCurdy (6), this fact has been successfully used as a guide to the recognition of cell injury when other methods have failed. It was therefore hoped that the fate of the leucocyte in the pneumonic exudate might be determined by this method.

In this experiment forty-one rabbits were used. Some of the animals received one, others two, and still others three intravenous injections of twenty cubic centimeters of a 1 per cent. trypan blue solution. In some cases the dye was given before the intratracheal inoculation of pneumococci, while in others the first dose of trypan blue was given as late as sixty-five hours after the injection of the organisms. The animals were killed at intervals varying from three hours to six and one-half days after the intratracheal injection of pneumococci.

The results of these experiments may be summarized as follows: As early as seven hours after the intratracheal inoculation of pneumococci an occasional polymorphonuclear leucocyte with its nucleus stained intensely blue occurs in the exudate. These apparently dead cells increase in number as the process advances but never form the majority of the cells of the exudate. At first these stained cells have distinctly polymorphous nuclei, but slowly the characteristic shape of the nucleus is lost and it is converted into a small, homogeneous, blue staining mass. Furthermore, as early as three hours after the process has begun, polynuclear cells containing blue granules in their cytoplasm are seen in the alveoli, bronchioles, and blood vessels.

These granules correspond in size, shape, and number to the amphophilic granules of the rabbit leucocytes, and occur in a large number of polynuclear cells at all stages of the disease. As the process advances, in addition to these granules, the cytoplasm occasionally contains within it much larger, irregular, blue masses.

Since vitally stained granules have never been described in polymorphonuclear leucocytes, these findings incited a more careful study of the polynuclear cells in the general circulation of otherwise

untreated, vitally stained animals, and in vitally stained animals in which pneumonia had been produced.

A large number of smears from the ear vein of both these types of animals was studied. The polynuclear cells in these smears were almost constantly unstained; very exceptionally one with blue granules occurred. The significance of this selective staining of the granules of the leucocytes in the pneumonic lung has not been determined.

From the experiments of Winternitz and Evans, it is known that the granules of the polymorphonuclear leucocytes stain with trypan blue when the cell is injured. This injury may be accomplished in many ways; *i. e.*, by mixing the blood with benzol, by pressing the cover-slip forcibly on a fresh blood smear, etc. It is possible, therefore, that the staining of the granules of the leucocytes in the pneumonic lung may result from some injury to the cell membrane. This does not seem probable, since cells are found with only part of their granules vitally stained. The only elements in the pneumonic exudate that stain vitally are these granules and the fibrin. The granules, although at first similar in size and shape to the normal granules of the leucocyte, may become larger and more irregular in shape. It is possible that the staining of these granules may be associated with some functional change within them perhaps related to fibrin formation.

#### THE CIRCULATION IN THE PNEUMONIC LUNG.

When pneumonia is produced in animals with vital stain in their circulation, the involved lung has a uniform blue color; if, however, the dye is injected sometime after the pneumonia is produced (twenty to sixty-five hours), pale gray consolidated areas occur in the otherwise densely blue stained, involved lobes. Such areas occur most frequently at the periphery of the lobe, but there is no uniformity in their distribution. Similar areas were observed in the pneumonic lungs of animals not vitally stained. Here they appear grayish white and are much less granular on section than the remainder of the consolidated lobe. The sharp demarcation of these wedge-shaped, infarct-like areas was so striking in the vitally stained animals that it led to the following study of the circulation of the lung in pneumonia.

The consensus of opinion concerning the progressive pallor of the pneumonic lung after the stage of engorgement is expressed in the more recent text-books as follows: "The gray color is caused partly by the large number of leucocytes in the exudate and partly by the poor supply of blood in the capillaries which are compressed by the ever increasing exudate" (7). "As the exudation increases in amount and the fibrin meshwork thickens, the air-sacs are greatly distended, the alveolar walls stretched, and the capillaries compressed so that they no longer appear engorged. As a result, the redness due to congestion and to some extent to hemorrhage fades and the lung passes into the stage known as grey hepatization" (8).

The purpose of this investigation was to determine, first, whether there is any impairment of the circulation in the pneumonic lung, and, secondly, if so, how it is brought about.

On Dec. 15, 1913, there came to autopsy a patient with lobar pneumonia involving the right middle and upper lobes. Three and a half hours after the patient's death the lungs were injected through the pulmonary artery, under a pressure of 120 mm. of mercury, with a Berlin blue gelatin solution (equal parts of 5 per cent. Berlin blue and 10 per cent. gelatin solution). After the injection the pulmonary artery was tied and the lungs were placed in 10 per cent. formalin over night. On section the following morning a striking picture presented itself. The consolidated area (right upper and middle lobes) was pale gray, whereas the non-consolidated area (lower lobe) was intensely blue. An extremely small amount of the blue solution had found its way into the vessels of the consolidated area. Four subsequent cases, treated in the same manner, yielded the same result. With the same method, a series of rabbit lungs, showing experimental lobar pneumonia, was injected immediately after the death of the animals and on section these likewise presented the same striking contrast.

Microscopic examination of the human and rabbit lungs entirely corroborated the macroscopic findings. Very little of the blue solution was found in the consolidated areas, whereas the blood vessels of the uninvolved portions were engorged with the blue injection mass.

These experiments prove that after death, under exactly the same conditions, the vascular bed in the consolidated area can be but imperfectly injected with the dye as compared with the uninvolved portions of the same lung. It was next necessary to determine whether this circulatory impairment could be explained by the increased intra-alveolar pressure due to the exudate.

Accordingly a normal rabbit was killed with ether and the lungs were removed. A cannula was inserted into the left bronchus and the left lung was distended with air so that its volume was considerably greater than if it had been the seat of lobar pneumonia. While the left lung was still so distended, an injection mass was forced through the pulmonary artery under a pressure of 120 mm. of mercury. There was apparently no difference macroscopically be-



tween the lobes in which the intra-alveolar pressure was very great and those in which it was slight, either in the rapidity of injection or the amount of dye present.

Microscopic sections also failed to show any difference in the quantity of blue on the two sides.

This experiment was followed by another in which the intra-alveolar pressure was increased by the introduction of a 10 per cent. gelatin solution into the left bronchus under a pressure of 120 mm. of mercury. The solution and the lungs were kept at body temperature during the injection. After the gelatin had been forced into the alveoli the lungs were placed in cold water to allow the gelatin mass to solidify, and, when this had taken place, the lungs were injected through the pulmonary artery with a Berlin blue gelatin mass in the manner above described.

On section gross and microscopic examination failed to reveal any difference between the two sides.

A similar experiment was performed, with freshly drawn rabbit blood to distend the alveoli, and the results of the subsequent vascular injection were identical with those obtained when air and gelatin were used to raise the intra-alveolar pressure.

These experiments prove that the impaired circulation in the pneumonic lung can not be due to the pressure of the exudate within the alveoli. An attempt was then made to determine what element or elements of the inflammatory exudate were responsible for this impairment of the circulation.

A number of rabbits was given benzol until their leucocytes were reduced to below 1,000 per cm., and then in each one 5 c.c. of a culture of pneumococci were injected as deeply into a bronchus as the catheter could be inserted. At the end of forty-eight hours the animals were killed with ether. They all showed lobar type of consolidation. With the same method described above the lungs were injected with Berlin blue gelatin.

On section it was found that the consolidated areas were for the most part grayish white in appearance, whereas the uninvolved lobes were colored deep blue. Microscopically the exudate in the consolidated areas was found to consist almost entirely of fibrin. In addition there was a small amount of serum, an occasional alveolar cell, but very few leucocytes. The vessels in these areas contained very little of the blue injection mass and corresponded in this respect to the vessels of the injected pneumonic lung in normal rabbits.

These experiments indicate that the fibrin is the important element in the interference of the circulation in the pneumonic lung, for pneumonia in aplastic animals is characterized by an exudate of fibrin with an almost total absence of cellular elements (9). An attempt was therefore made to produce pneumonia in animals in

which the fibrin formation had been inhibited. This was accomplished through the use of phosphorus and chloroform.<sup>1</sup>

A rabbit was injected subcutaneously with 0.8 c.c. and 0.4 c.c. of an old solution of phosphorus in olive oil, together with 0.9 c.c. of chloroform in 30 per cent. alcohol intragastrically, on two successive days. Twenty-four hours later the clotting time of the blood was found to be greatly delayed, and the resulting clot very soft and jelly-like. Then 5 c.c. of a twenty-four hour culture of pneumococci in pig serum broth were injected as deeply into a bronchus as the catheter could be inserted. The animal died in seventeen and one half hours, and at autopsy showed consolidation of practically the entire left lower lobe. Berlin blue gelatin was then injected through the pulmonary artery.

On section the consolidated lobe was found to be almost as uniformly blue as the uninvolved lobes. Microscopic section showed that the exudate contained an abundance of cells but very little fibrin. Furthermore, the vessels of the consolidated area contained a much greater amount of dye than had been observed in any of the pneumonic lungs described above.

These experiments demonstrate that the fibrin is responsible for the impairment of the circulation of the lung in pneumonia. A study of the distribution of the fibrin was made to determine how it produced the obstruction.

Microscopic examination of the pneumonic lungs from the above experiments showed a large number of fibrin plugs in the capillaries and even in the larger vessels. The only exception was the consolidated lung of the animal that had received chloroform and phosphorus. The fibrin thrombi have been observed by others (10, 11), but they were considered to be a postmortem, or at most an agonal, phenomenon and of no significance in the pneumonic process.

That they occur early in the course of pneumonia can be demonstrated from the experiment with trypan blue. This is a most readily diffusible dye and reaches all areas whose blood supply is not impaired. When pneumonia is produced in animals in whose circulation this dye is present, the consolidated area becomes uniformly stained. If the dye is injected twenty hours after the pneumonia is produced, the consolidated area is not uniformly stained, and, as has been said, it may show sharply demarcated and absolutely unstained areas.

It may, therefore, be concluded that fibrin plugs form in the capillaries early in pneumonia and that these impair the circulation in the consolidated lung. The distribution of the fibrin plugs in the ves-

<sup>1</sup> We are indebted to Drs. Whipple and Goodpasture for aid in this experiment.

sels in the pneumonic lung of man is relatively uniform throughout, and by their interference with the circulation they probably cause the pallor of the lung in gray hepatization. In the pneumonic lung of the rabbit the distribution of these plugs is not uniform; they may be so extensive that there is an absolute loss of circulation in local areas. Such areas are likely to undergo necrotization and probably result in the minute abscesses which have been found repeatedly in rabbits' lungs long after the pneumonic process has subsided.

The importance of this wide-spread formation of fibrin plugs in the vessels in the pneumonic lung is at once evident. The experiments of Opie (12) have shown the presence of an antitryptic substance in the serum which prevents the autolysis of leucocytes and the digestion of fibrin by leucocytes. The leucocytes alone digest this material rapidly. Therefore, with an interference of the circulation the leucocytes of the exudate are much less influenced by the action of the serum which reaches them in minimum amount, and as a result autolysis takes place in the exudate just as it would if the pneumonic lung were placed in the thermostat. There can be no doubt that this interference in the circulation in the pneumonic lung is of great importance in the resolution of the exudate.

It is further probable that the interference of the circulation in the pneumonic lung is responsible for the restricted action of immune serum in this disease. The experiments of Cole (13) have shown that the septicemia of pneumonia, and consequently the fatal outcome of the disease, may be averted in many cases by the intravenous administration of the specific immune serum, but the progress of the local lesion remains uninfluenced. This must be expected since, on account of the impaired circulation, the immune serum can not reach the local lesion in sufficient amounts to influence its progress. It is evident that this same criticism would apply to any therapeutic measure so administered, and it therefore seemed advisable to ascertain whether the consolidated area could be impregnated in some other way.

The following experiments were carried out to see whether the consolidated area could be stained by an intratracheal injection of a dye.

Pneumonia was produced in a rabbit, and forty-eight hours later, under ether anesthesia, the anterior thorax was removed, and artificial respiration by the Meltzer method of intratracheal insufflation was instituted. Respiration was interrupted long enough to allow the injection of 10 c.c. of a 1 per cent. solution of trypan blue into the consolidated lung through a catheter inserted into its bronchus. The animal was then killed by ether.

On section of the consolidated lung it was found to be uniformly and intensely stained. Microscopic section showed the presence of the dye in practically all the alveoli localized in the same elements which are stained after intravenous injection of the dye.

This experiment was repeated with a similar result. It may be said, therefore, that the presence of fibrin plugs throughout the capillary bed of the pneumonic lung interferes greatly with the penetration even of such a diffusible substance as trypan blue, when this drug is injected intravenously, but the exudate offers no serious obstruction to the penetration of the dye into the alveoli when it is injected intrabronchially.

The method of intrabronchial treatment of pneumonia therefore suggests itself.

#### CONCLUSIONS.

1. Dead leucocytes are constantly found in the pneumonic exudate. They rapidly undergo disintegration. Up to the seventh day they do not form the majority of the cells of the exudate.

2. Polymorphonuclear leucocytes with vitally stained granules are present in the exudate, vessels, and interstitial tissue of the lung in experimental pneumonia, but they are not demonstrable in the general circulation in the same animals.

3. There is a marked impairment of the circulation in the pneumonic lung.

4. The increase in the intra-alveolar pressure exerted by the exudate has no influence upon the circulation.

5. The impaired circulation results from the wide distribution of capillary fibrin thrombi. In man these are, as a rule, distributed with relative uniformity. In the rabbit this is not usually the case. The thrombi are much more abundant in some areas and may lead to localized necrosis.

6. The impairment of the circulation is of importance in bringing about resolution. Only enough blood is allowed to seep through

the vessels to nourish the alveolar walls. Consequently very little serum escapes into the alveoli and the autolysis of the exudate by the leucocytes is unhindered.

7. The impairment of the circulation in the pneumonic lung seriously interferes with the action of intravenous therapy upon the local lesion.

8. The exudate in the pneumonic lung can be readily impregnated with a dye injected intrabronchially. This suggests a method of administration of therapeutic agents in pneumonia.

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## STUDIES UPON EXPERIMENTAL PNEUMONIA IN RABBITS.

### IX. THE PART OF THE LEUCOCYTE IN THE IMMUNITY REACTION.\*

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It has been shown that pneumonia is much more fatal in aplastic than in normal rabbits and that the latter are less resistant to the disease than animals in which a leucocytosis has been artificially produced (1). Recently Gay (2) has reported a hyperleucocytosis following the intravenous injection of antigen in immunized animals, thus indicating the importance of the leucocyte in the immunity reaction as well as in the initial defense. It was therefore determined to study the part of the leucocyte in the immunity reaction.

In the following paper the effect of the intravenous injection of pneumococci in normal, aplastic, and in actively and passively immunized normal and aplastic rabbits will be presented.

#### METHOD.

It is possible to follow the extent of the bacteriemia in pneumonia by determining the number of organisms in relation to the number of red blood cells. This method is not satisfactory when the organisms are present in small numbers; then they can not be counted accurately or are entirely overlooked. Therefore the following method of making blood cultures was devised. The animals' ears were shaved and the skin was sterilized with 95 per cent. alcohol. One of the larger veins was then punctured with a sterile needle, and a loop of blood was immediately smeared over the surface of a rabbit blood agar slant. This method allows of rapidly repeated cultures and does not interfere with the condition of the animal.

All the cultures in the experiments to be reported were made in the manner described. In all the same amount of blood was taken. The tubes were incubated for forty-eight hours before observations were recorded.

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THE FATE OF INTRAVENOUSLY INJECTED PNEUMOCOCCI AND ITS RELATION TO THE LEUCOCYTIC REACTION IN NORMAL ANIMALS.

Fourteen normal rabbits were given an overwhelming dose of pneumococci intravenously. Blood cultures and smears were made at short intervals until the animals died. In several a number of leucocyte counts were made. The results were similar in all cases (table I).

TABLE I.

Date (1914).	Time.	Procedure.	No. of pneumococci in blood smears.	No. of colonies in blood cultures.	White blood count per c.mm.	Result.
June 3	9 A.M.	.....	.....	.....	11,700	.....
	10 A.M.	8 c.c. pneumo- cocci intra- venously	.....	.....	.....	.....
	10.10 A.M.	.....	Too few to count	Innumerable	.....	.....
	11 A.M.	.....	Too few to count	Innumerable	.....	.....
	3 P.M.	.....	400,000 per c. mm.	Innumerable	.....	.....
	5 P.M.	.....	800,000 per c. mm.	Innumerable	.....	.....
	7 P.M.	.....	1,335,000 per c. mm.	Innumerable	2,200	.....
	9 P.M.	.....	3,800,000 per c. mm.	.....	3,900	.....
	1 A.M.	.....	.....	.....	4,400	.....
June 4	10 A.M.	.....	.....	.....	.....	Death.

It may be seen from table I that following an intravenous injection of a lethal dose of pneumococci in a normal rabbit the organisms increase steadily in number until the animal dies. Further, there is a marked and persistent fall in the number of leucocytes in the peripheral circulation.

THE FATE OF INTRAVENOUSLY INJECTED PNEUMOCOCCI AND ITS RELATION TO THE LEUCOCYTIC REACTION IN APLASTIC ANIMALS.

Experiments with several rabbits in which the leucocytes had been reduced to below 1,000 per cubic millimeter by the daily injection of benzol showed that these animals are more susceptible than normal rabbits to the intravenous injection of pneumococci. No special experiments were undertaken to determine the leucocytic reaction, as the result of such a procedure seemed sufficiently evident from previous experiments in which pneumococci had been injected intratracheally in aplastic animals. Here a bacteriemia developed while the number of leucocytes remained uninfluenced.

THE FATE OF INTRAVENOUSLY INJECTED PNEUMOCOCCI AND ITS  
RELATION TO THE LEUCOCYTIC REACTION IN PASSIVELY  
IMMUNIZED RABBITS.

Three rabbits were passively immunized and then injected intravenously with an overwhelming dose of homologous pneumococci. Blood cultures were taken at short intervals over a period of twenty-seven to seventy-two hours. The results in all were practically identical (table II).

TABLE II.

Date (1914).	Time.	Procedure.	No. of colonies in blood cultures.	Result.
June 8	9 A.M.	20 c.c. immune serum	.....	.....
	10 A.M.	10 c.c. virulent pneumococci intravenously	.....	.....
	10.05 A.M.	.....	Very many	.....
	10.30 A.M.	.....	None	.....
	11 A.M.	.....	None	.....
	2 P.M.	.....	None	.....
	8.30 P.M.	.....	None	.....
June 9	1 P.M.	.....	None	Recovery.

Table II shows that, following the intravenous injection of a lethal dose of pneumococci in a passively immunized rabbit, the organisms are present in large numbers for the first five minutes, but are no longer demonstrable in the circulation thirty minutes after the injection.<sup>1</sup>

<sup>1</sup>In the other two rabbits in this series the time of disappearance of the organisms was one hour and three hours, respectively. All three animals recovered completely.



THE FATE OF INTRAVENOUSLY INJECTED PNEUMOCOCCI AND ITS  
RELATION TO THE LEUCOCYTIC REACTION IN ACTIVELY  
IMMUNIZED RABBITS.

In this experiment fifteen rabbits were used. The animals received a number of gradually increasing doses of pneumococci until they possessed a decided immunity. They were then injected intravenously with a lethal dose of pneumococci of the same strain. Blood cultures were taken and the leucocytes counted at short intervals after the injection. The chart of animal 2 (table III) is characteristic of this group.

TABLE III.

Date (1914).	Time.	Procedure.	No. of colonies in blood culture.	White blood count per c.mm.	Result.
Apr. 17 to May 23	.....	Gradually increasing doses of pneumococci intravenously from 0.5 c.c. to 9.0 c.c.	.....	.....	.....
June 3	9.15 A.M.	.....	.....	10,800	.....
	10.15 A.M.	8 c.c. of pneumococci in- travenously	.....	.....	.....
	10.20 A.M.	.....	Innumerable	.....	.....
	10.30 A.M.	.....	Very many	.....	.....
	10.45 A.M.	.....	Many	.....	.....
	11.15 A.M.	.....	50	.....	.....
	12.15 P.M.	.....	12	.....	.....
	2.15 P.M.	.....	None	.....	.....
	4.15 P.M.	.....	None	.....	.....
	7.15 P.M.	.....	None	23,300	.....
	10.15 P.M.	.....	None	37,300	.....
June 4	1.15 A.M.	.....	None	27,900	.....
	10.15 A.M.	.....	None	.....	Recovery.

Table III shows that in actively immunized rabbits the injection of a lethal dose of pneumococci is followed by a rapid disappearance of the organisms from the circulation, and that there is a decided leucocytosis reaching its maximum about twelve to fifteen hours after injection. This leucocytosis occurs many hours after the disappearance of the organisms from the circulation.

THE FATE OF INTRAVENOUSLY INJECTED PNEUMOCOCCI AND ITS  
RELATION TO THE LEUCOCYTIC REACTION IN APLASTIC  
PASSIVELY IMMUNIZED RABBITS.

Eight rabbits were given daily doses of benzol subcutaneously until the number of their leucocytes was below 1,000 per c.mm. At this stage they were passively immunized and then injected with a lethal dose of the homologous pneumococci. Blood cultures were taken at short intervals, and in one animal leucocyte counts were made before and after the injection of the organisms. All these animals reacted identically (table IV).

TABLE IV.

Date (1914).	Time.	Procedure.	No. of colonies in blood cul- tures.	White blood count per c.mm.	Result.
May 27 to	.....	Daily subcutaneous in- jections of benzol	.....	.....	.....
June 2	.....	.....	.....	340	.....
June 3	8.30 A.M.	.....	.....	.....	.....
	8.45 A.M.	10 c.c. immune serum intravenously	.....	.....	.....
	9.30 A.M.	10 c.c. immune serum intravenously	.....	.....	.....
	9.45 A.M.	8 c.c. pneumococci intra- venously	.....	.....	.....
	9.50 A.M.	.....	Many	.....	.....
	10.15 A.M.	.....	36	.....	.....
	10.45 A.M.	.....	6	.....	.....
	11.45 A.M.	.....	1	.....	.....
	1.45 P.M.	.....	1	.....	.....
	3.15 P.M.	.....	6	160	.....
	7.45 P.M.	.....	None	80	.....
June 4	9.45 A.M.	.....	Very many	.....	.....
June 5	3.45 A.M.	.....	Innumerable	.....	Death.

Table IV shows that after the intravenous injection of a lethal dose of pneumococci in passively immunized aplastic rabbits there is a rapid disappearance of the organisms from the circulation; within two to four hours they have practically disappeared. The disappearance of the organisms from the blood is, however, only temporary. The bacteriemia recurs and there is a steady increase in the number of organisms until the animal dies. In addition, it may be noted that the injection of the pneumococci in these animals is not followed at any time by an increase in the number of leucocytes in the peripheral circulation.

THE FATE OF INTRAVENOUSLY INJECTED PNEUMOCOCCI AND ITS  
RELATION TO THE LEUCOCYTIC REACTION IN ACTIVELY

IMMUNIZED APLASTIC RABBITS.

Three animals were actively immunized in the same manner as those previously mentioned. When they possessed a decided immunity they were given daily subcutaneous injections of benzol until their leucocytes were reduced to below 1,000 per c.mm. At this stage they were each injected intravenously with a lethal dose of pneumococci of the same strain used before, and blood cultures were taken at short intervals thereafter. In these animals leucocyte counts were also made several times after the injection of the organisms.

The reaction in all cases was the same and is illustrated by table V.

TABLE V.

Date (1914).	Time.	Procedure.	No. of colonies in blood cul- tures.	White blood count per c.mm.	Result.
Apr. 17 to May 23		Gradually increasing doses of pneumococci injected intravenously up to 9 c.c.			
May 25 to June 2		Daily subcutaneous in- jections of benzol			
June 3	8.50 A.M.	8 c.c. pneumococci intra- venously			
	9.00 A.M.		Innumerable	280	
	9.20 A.M.		Innumerable		
	9.50 A.M.		36		
	10.20 A.M.		3		
	11.20 A.M.		1		
	1.20 P.M.		None		
	7.20 P.M.		None	380	
	9.20 P.M.		None	560	
June 4	12.20 A.M.		None	500	
	9.20 A.M.		None	380	
June 5	3.20 A.M.		None	580	Recovery.

Table V shows that, following the intravenous injection of a lethal dose of pneumococci in actively immunized aplastic animals, there is a permanent disappearance of the organisms from the circulation, and the rabbit recovers.

In these actively immunized aplastic animals there is no increase in the number of leucocytes at any time after the injection of the pneumococci.

SUMMARY AND DISCUSSION.

Following the intravenous injection of an overwhelming dose of pneumococci in normal animals there is a rapidly increasing bacteremia which reaches its maximum with the death of the animal.

Immunized animals, whether the immunity is active or passive, whether the animals have their leucocytes, or whether these have been destroyed by benzol, react differently. They have in common the ability to cause the organisms to decrease rapidly in number, and, as far as could be determined by the methods used, to disappear absolutely in a very short time from the circulation. This may be called the immediate reaction.

In actively and passively immunized normal rabbits the disappearance of the organisms from the blood is followed by their destruction in the body and the ultimate recovery of the animal. This same result occurs also in actively immunized rabbits deprived of their polymorphonuclear leucocytes by benzol. On the contrary, in passively immunized rabbits, deprived of their leucocytes in the same way, the immediate disappearance of the organisms from the circulation is followed, after a lapse of from six to twenty-four hours, by a recurrent, gradually increasing bacteriemia and the death of the animal.

It is evident that the immediate reaction is no index of the ultimate result.

This finding, that the result of the intravenous injection of an overwhelming dose of pneumococci in immunized animals may be divided into two stages, immediate and ultimate, has been utilized in the interpretation of the experiments that have been reported above.

1. *Normal Rabbits.*—In the normal rabbit the injection of a lethal dose of pneumococci is followed by the same immediate and ultimate response. The animal develops a rapidly progressing bacteriemia and dies.

2. *Passively Immunized Normal Rabbits.*—A diametrically opposed finding is obtained when the animals are passively immunized. Then following the intravenous injection of pneumococci the organisms rapidly disappear from the circulating blood and the animal recovers. The only apparent difference between these two groups of experiments is the introduction of a relatively small quantity of immune serum. Tentatively, therefore, it may be concluded that the immune serum is responsible both for the immediate and ultimate reaction in this case.

3. *Passively Immunized Aplastic Rabbits*.—A normal rabbit that has been previously benzolized, and in this way deprived of its myeloid elements, can not be successfully immunized. Here the immune serum injected even in excessive quantities is followed by the immediate reaction, but the ultimate result is entirely different, —the animal dies.

It may be concluded, therefore, that the serum is the potent factor in bringing about the immediate disappearance of the organisms from the circulation.

Furthermore, it is evident that the white blood cells of the myeloid tissue are necessary in order that passively immunized animals may recover following the introduction of a lethal dose of pneumococci. So far it seems that two elements are essential in the immunity process; *i. e.*, immune bodies and white blood cells.

Corroborative evidence of the importance of the white blood cell in this reaction is offered by the hyperleucocytosis which follows the injection of antigen in actively immunized animals (Gay). This hyperleucocytosis occurs in actively immunized rabbits injected with pneumococci, after the organisms have disappeared from the circulation. It occurs at about the same time that the septicemia recurs in passively immunized aplastic rabbits.

4. *Actively Immunized Aplastic Rabbits*.—The experiments with actively immunized aplastic rabbits complicate the conception of the part of the white blood cells in the immunity process. When actively immunized, benzolized animals are injected with a lethal dose of pneumococci, the immediate reaction occurs just as in the passively immunized aplastic animal. Available antibodies are present and cause the disappearance of the organisms from the circulation. The ultimate reaction differs from that in the passively immunized aplastic animal. The latter develops a recurrent bacteriemia and dies. The actively immunized aplastic animal recovers.

It would seem that the white blood cell is no longer necessary in an animal that has been actively immunized, but that it must be present for the passive protection of the animal.

The ultimate reaction in immunized rabbits seems to be dependent upon some action of the white blood cells. In the passively immunized animal this may occur at the time of, or following, the im-

mediate reaction; and if it can not occur, owing to the absence of the white blood cells, the animal subsequently dies. In the actively immunized aplastic rabbit this action has apparently occurred at a previous time and is sufficiently developed to protect the animal even though the leucocytes have been destroyed.

It may be assumed that the function of the white blood cell is exercised not directly, but perhaps indirectly, by some influence exerted on other body cells. This interrelation or interaction between the white blood cells and other cells in the body constitutes a third factor essential to the ultimate protection of the animal.

Additional evidence of this action of the white blood cell is furnished by further experiments with actively immunized aplastic rabbits. Several immunized rabbits were benzolized and then injected with a lethal dose of pneumococci. The results were the same as those indicated above. The organisms disappeared rapidly from the circulation and the animals recovered.

An interval of five days was allowed to elapse and the animals were injected again with the same amount of pneumococci. This time the reaction was different. The organisms disappeared rapidly from the circulation, but there was a recurrent bacteriemia and the animals died. In these animals the favorable ultimate result after the first inoculation of a lethal dose of pneumococci probably depended upon the presence of the immune bodies and the third factor mentioned above. After the subsequent inoculation of pneumococci the immediate reaction was followed by the recurrence of the bacteriemia and the death of the animals. It may, therefore, be inferred that the third factor present at the time of the first injection had been destroyed, and, owing to the absence of white blood cells at the time of the subsequent inoculation, it was not formed again.

It may be concluded that there are at least three elements necessary in the immunization process: (1) immune bodies, (2) white blood cells, and (3) a third factor which is dependent for its existence upon the presence of white blood cells at the time of inoculation of the pneumococci. Furthermore, this third factor may be removed if the animal is inoculated at a time when it is aplastic.

#### CONCLUSIONS.

1. The result of the intravenous injection of pneumococci in immunized rabbits may be divided into two stages,—immediate and ultimate.

2. The immediate reaction is not decisive of the ultimate result.

3. The immunity process seems to be dependent upon at least three factors: immune bodies, white blood cells, and a third factor which is dependent for its existence upon the presence of the white blood cells at the time of the inoculation of the pneumococci.

This third factor may be removed by rendering an immunized normal rabbit aplastic and then injecting it with a minimal lethal dose of pneumococci.

The result of the injection of this antigen into immune rabbits varies according to the presence of the three factors mentioned above. The immune bodies cause an immediate disappearance of the organisms from the circulation. The third factor causes the permanent absence of the organisms from the circulation and the recovery of the animal. The white blood cells seem to be essential for the production of this third factor.

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## THE INFLUENCE OF EPINEPHRIN UPON THE CORONARY CIRCULATION OF THE MONKEY.\*

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### PLATE 22.

Epinephrin (adrenalin) undoubtedly dilates the coronary arteries of the animals that are usually made the objects of laboratory investigation. That a better blood supply should thus be afforded the heart by a hormone which arouses a marked increase in activity seems a suitable provision of nature.

The most important ways in which epinephrin may influence the coronary circulation probably are: (1) directly, by acting upon the myoneural junctions of the vessels themselves; (2) indirectly, (a) by changes in the activity of the heart, which may affect the vessels by the production of metabolites (Markwalder and Starling<sup>1</sup>), or mechanically, (b) by changes in the general arterial pressure. (c) by possible actions on the central nervous system.

As has been shown by the classical studies of Langley and his co-workers, the direct action of epinephrin upon the coronary vessels depends presumably upon their innervation. In the laboratory animals thus far investigated these arteries appear to be innervated by dilators of true (thoracolumbar) sympathetic origin, because epinephrin increases the coronary flow in perfused isolated hearts (whether active or at rest) and causes a relaxation in isolated coronary strips or rings.

Electrical stimulation of the accelerator nerve was found by Maass<sup>2</sup> to increase the coronary flow in the cat, thus indicating in a more direct manner the existence of coronary dilators of sympathetic

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<sup>1</sup> Markwalder, J., and Starling, E. H., *Jour. Physiol.*, 1913, xlvii, 275.

<sup>2</sup> Maass, P., *Arch. f. d. ges. Physiol.*, 1899, lxxiv, 281.



origin in this animal. This has recently been corroborated by Morawitz and Zahn.<sup>3</sup>

These authors were impressed by the fact that for the study of many questions relating to the coronary circulation the intact animal is best adapted. The indirect factors in the living animal, of course, modify the influence of the direct. This had been recognized previously by Bond,<sup>4</sup> who worked on the coronary circulation of intact animals in 1910.

Morawitz and Zahn inserted a catheter through the right auricle into the coronary sinus of the hirudinized cat or dog. After measuring with a suitable flow recorder the coronary blood thus collected, they returned it through a slow feeding burette into the jugular vein. Records were obtained of the increase in coronary flow resulting from raising the general arterial pressure in various ways.

The effect of epinephrin upon the coronary flow was then shown to be one of extreme augmentation, even out of proportion to the increase in general arterial pressure. This indicated again the influence of other factors such as increased metabolites or an actively stimulated vasodilator mechanism. Similar, although less marked, effects were obtained by subcutaneous injections of epinephrin.

Angina pectoris was then treated in this manner by Büdingen<sup>5</sup> at the instigation of Morawitz and Zahn. No positive results being obtained, these authors were led to remark that it is by no means clearly established that anginal attacks are due to coronary spasm.

On the other hand, one of us<sup>6</sup> obtained evidence three years ago that the action of epinephrin in man differs from its action in the usual laboratory animals as regards the coronary arteries. It was demonstrated that isolated rings of these arteries obtained from fresh cadavers respond to epinephrin by contraction only. These experiments were controlled by many upon coronary rings of the calf, sheep, and pig, in which was constantly seen the usual relaxation. The conclusion was drawn that epinephrin constricts the

<sup>3</sup> Morawitz, P., and Zahn, A., *Zentralbl. f. Physiol.*, 1912-13, xxvi, 465; *Deutsch. Arch. f. klin. Med.*, 1914, cxvi, 364.

<sup>4</sup> Bond, G. S., *Jour. Exper. Med.*, 1910, xii, 575.

<sup>5</sup> Büdingen's results are reported by Morawitz and Zahn, *Deutsch. Arch. f. klin. Med.*, *loc. cit.*, p. 388.

<sup>6</sup> Barbour, H. G., *Jour. Exper. Med.*, 1912, xv, 404.

human coronary vessels and that they are therefore supplied, presumably, with vasoconstrictors of true sympathetic origin.

Morawitz and Zahn express themselves as willing to entertain this view as an alternative explanation of Büdingen's negative results with epinephrin in angina pectoris.

The constricting influence of epinephrin upon the human coronaries has, however, been doubted by some orthodox students of epinephrin; for example, Park.<sup>7</sup> With the hope of obtaining corroborative evidence from the monkey the following work was undertaken.

#### METHOD.

The experiments were made upon normal members of the species *Macacus rhesus*, the control animals being normal rabbits. In these two classes of animals we sought a variation in the action of epinephrin upon the coronary vessels themselves. We selected, therefore, a method which excluded some of the above mentioned indirect factors, employing isolated hearts. For this purpose the perfusion apparatus described by Locke and Rosenheim<sup>8</sup> was found well adapted.

The animals were decapitated and the blood was collected into a vessel containing 0.02 to 0.04 of a gram of hirudin dissolved in 50 cubic centimeters of Locke solution. The blood mixture was filtered and diluted further when necessary to make about 150 cubic centimeters. The blood constituted one-third to one-half of the total mixture.

The heart was immediately excised and connected with the aortic cannula of the perfusing system. After thorough irrigation of the coronary vessels with Locke solution, the blood mixture was transferred to the reservoir of the apparatus and the experiment begun.

In order to obtain a uniform record of the coronary flow it was found advisable to suspend the heart, apex upward, thus preventing the accumulation of blood in the right auricle and ventricle, which leads to an irregular outflow from the beating heart. The heart was held in this position, below, by means of a fixed, curved aortic cannula and above by a thread sutured to the apex of the ventricles.

The rate of coronary flow was measured with Condon's<sup>9</sup> tipper

<sup>7</sup> Park, E. A., *Jour. Exper. Med.*, 1912, xvi, 532.

<sup>8</sup> Locke, F. S., and Rosenheim, O., *Jour. Physiol.*, 1907-8, xxxvi, 205.

<sup>9</sup> Condon, N. E., *Jour. Physiol.*, 1913, xlv, p. xlv.

recorder. The perfusion fluid unit was constant during each experiment. It varied somewhat in the series, averaging about 2 cubic centimeters. The injections were made obliquely through the rubber tubing at a point a few centimeters above the aortic cannula. To avoid changes in arterial pressure this was done slowly and with the needle directed against the current of the perfusing fluid.

As shown in the tables, perfusion pressures of 50, 75, and 100 millimeters of mercury were employed. The perfusion fluid was maintained at a constant temperature of 38° C.

Commercial adrenalin chloride was used throughout the work.

#### RESULTS.

The experiments are summarized in tables I and II. In these it will be noted that no time has been allowed between the injection and the arrival of epinephrin at the coronary vessels. Thus the degree of response is in all cases underestimated. A further source of underestimation in the tables is the fact that the three minute interval chosen often expired before the full effect of the drug was seen. It suffices, however, for demonstrating the main fact, which is a qualitative one.

A better conception of the character of the individual results is obtained from the reproduced tracings. In these the upper line records the rate of coronary flow, the lower the time in five second intervals.

*Rabbit Controls.*—Seven experiments were made upon two rabbit hearts. The first table shows that all doses employed, varying from 0.025 to 0.25 of a milligram, gave constantly an increase in coronary flow.

TABLE I.  
*. Isolated Rabbit Hearts.*

Animal.	Experiment.	Perfusion pressure.	Epinephrin.	Units of perfusion fluid in 3 min.	
				Before epinephrin.	After epinephrin.
Rabbit 1 (3,300 gm.).....	1	50 mm. Hg.	0.00025 gm.	3.2	5.4
	2	50 mm. Hg.	0.0001 gm.	3.0	3.8
	3	50 mm. Hg.	0.00025 gm.	1.8	2.9
Rabbit 2 (1,600 gm.).....	4	100 mm. Hg.	0.0001 gm.	7.2	13.3
	5	100 mm. Hg.	0.000025 gm.	3.2	3.8
	6	100 mm. Hg.	0.0001 gm.	3.1	4.3
	7	50 mm. Hg.	0.0001 gm.	1.5	3.2

Figure 1 is a sample record from a rabbit's coronary flow (experiment 1). A pre-injection period is shown, followed by the increase produced by epinephrin and a partial return to normal.

*Monkeys.*—That constriction of the coronaries by epinephrin is as constant in the isolated monkey heart as dilatation of the coronaries in the rabbit heart is shown by table II.

TABLE II.  
*Isolated Monkey Hearts.*

Animal.	Experiment.	Perfusion pressure.	Epinephrin.	Units of perfusion fluid in 3 min.	
				Before epinephrin.	After epinephrin.
Monkey 1, ♂ (1,400 gm.)..	8	50 mm. Hg.	0.00025 gm.	9.3	7.8
	9	50 mm. Hg.	0.00025 gm.	6.0	5.2
Monkey 2, ♀ (1,200 gm.)..	10	75 mm. Hg.	0.0005 gm.	13.4	13.1
	11	50 mm. Hg.	0.0005 gm.	5.8	5.4
	12	50 mm. Hg.	0.001 gm.	4.9	4.5
	13	50 mm. Hg.	0.002 gm.	4.2	3.5
	14	50 mm. Hg.	0.002 gm.	10.0	3.6
	15	75 mm. Hg.	0.002 gm.	3.0	1.8
Monkey 3, ♂ (1,800 gm.)..	16	100 mm. Hg.	0.000025 gm.	5.8	4.7
	17	100 mm. Hg.	0.0001 gm.	3.7	3.2
	18	100 mm. Hg.	0.0001 gm.	1.8	1.4
	19	100 mm. Hg.	0.001 gm.	4.2	3.8
	20	100 mm. Hg.	0.0001 gm.	8.2	6.3
	21	100 mm. Hg.	0.002 gm.	6.7	4.3

Fourteen experiments were made upon the hearts of three monkeys, the doses varying from 0.025 to 2 milligrams.

Figure 2 (experiment 8) is from the coronary flow of a male monkey, weighing 1,400 grams, a few minutes after removal of the heart from the body. The beat was active and regular; the heart rate per ten seconds is indicated by the numbers written on the tracing between the flow and time records. By comparison of the eight minute periods before and after the injection of 0.00025 of a gram of epinephrin, it will be seen that 26 perfusion units before were succeeded by 21 units after. The heart rate increased from 84 to 108 as a result of the injection. The tracing shows also the beginning of recovery from the coronary constriction.

Figure 3 illustrates the coronary flow of a female monkey, weighing 1,200 grams, several hours after removal of the heart, being the last of the series on the animal (experiment 14). Two milligrams of

epinephrin were given. The heart had ceased to beat for some time, but had been active during the earlier experiments tabulated from this animal.

The third monkey was a male weighing 1,800 grams. Experiment 17, from this monkey, is illustrated by figure 4. The heart was beating rhythmically before this experiment but within a minute after the epinephrin injection went into partial block, from which it never recovered. After a number of further injections with epinephrin had all yielded constriction, 0.025 of a cubic centimeter of amyl nitrite was injected. The marked flow increase illustrated in figure 5 (experiment 22) was the result. This indicates that the coronaries of the monkey, as of other animals, respond to amyl nitrite by dilatation.

#### DISCUSSION.

Our results greatly enhance the value of Barbour's earlier experiments upon isolated human coronaries, by annulling all the objections to the theory of coronary constriction by epinephrin in man, which are based upon analogy with other classes of mammalia. There exists, we believe, between certain primates, on the one hand, and certain of the lower mammalia on the other, a previously unsuspected difference in innervation. It has been detected by means of an agent which is already known to exhibit variations in its action upon the uterus of different species. Examples of this, with the literature, will be found in a recent paper by Gunn.<sup>10</sup>

As regards man, the constricting influence of epinephrin upon the coronaries, while having a possible bearing upon the therapy of anginal attacks, may also prove to be related to their etiology. In view of the work of Cannon<sup>11</sup> and others upon the discharge of epinephrin into the blood stream in certain emotional states, an explanation may be found for the frequent association of anginal attacks with excitement.

A factor which is common to all species and which operates against coronary constriction has been purposely omitted from the present work; namely, the decided increase in arterial pressure.

<sup>10</sup> Gunn, J. A., and Gunn, J. W. C., *Jour. Pharmacol. and Exper. Therap.*, 1914, v, 527.

<sup>11</sup> Cannon, W. B., and de la Paz, D., *Am. Jour. Physiol.*, 1911, xxviii, 64.

With stationary perfusion pressures of 50, 75, or 100 millimeters of mercury, which were never changed during an epinephrin experiment, no essential differences were seen in the results. When the pressure was raised or lowered between two experiments a corresponding increase or diminution in coronary flow was, of course, noted. Central effects of epinephrin, which are presumably of minor importance, have also been excluded from this work.

The factor of heart activity is important and has been dealt with to some extent. The earlier experiments upon each animal were all made upon actively beating hearts; many of the later ones upon hearts which were quiescent and remained so. We can say with certainty that the action of epinephrin upon the coronary flow was constant under both sets of conditions. We gained further the impression that the constrictor effect was more marked in resting than in active hearts, which may have been due to the lack of the non-volatile metabolites referred to by Starling and Markwalder. The variation in dosage in the present work precludes decision in this matter.

The dose was varied in the monkey from 0.025 of a milligram, which was ineffective in one experiment (not tabulated), up to two milligrams. Qualitatively our results were constant. The data are not sufficient to warrant quantitative deductions.

#### CONCLUSIONS.

Decrease in coronary flow was the constant response of freshly isolated monkey hearts to epinephrin. These hearts were perfused with autogenous hirudinized blood diluted with Locke solution. The results were constant at high or low perfusion pressures, in beating or resting hearts, and with all adequate doses. Increased coronary flow was obtained constantly in rabbit hearts under identical conditions.

In the light of previous work upon isolated human coronary arteries, the general conclusion is drawn that, while actively dilating the coronary vessels in the dog, cat, rabbit, ox, sheep, and pig, epinephrin constricts the coronary vessels in man and the monkey.

The coronary arteries of the last two species are presumably supplied with constrictor nerves of true sympathetic (thoracolumbar) origin.



FIG. 1.



FIG. 2.



FIG. 3.



FIG. 4.



FIG. 5.

(Barbour and Prince: Influence of Epinephrin upon Monkey.)





EXPLANATION OF PLATE 22.

Records of coronary perfusion. Upper line = perfusion units; lower line = time in five second intervals.

FIG. 1. Rabbit 1, 0.25 mg. of epinephrin.

FIG. 2. Monkey 1, 0.25 mg. of epinephrin. The numbers indicate the heart rate per ten seconds.

FIG. 3. Monkey 2, 2 mg. of epinephrin.

FIG. 4. Monkey 3, 0.1 mg. of epinephrin.

FIG. 5. Monkey 3, 0.025 c.c. of amyl nitrite.

## A TEST FOR ANTITHROMBIN IN THE BLOOD.\*

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The part that antithrombin plays in the human blood in inhibiting coagulation is a question concerning which there is a decided difference of opinion. Although it is admitted generally by physiologists that antithrombin is present in the circulating blood, there is a marked divergence as to the significance and importance which should be accorded it. On the one hand, we note that Morawitz, whose theory of coagulation has gained wide acceptance, assigns to antithrombin no function in the theory of coagulation which he has elaborated. This omission seems to be a weak link in the chain which he has constructed, as any theory is necessarily incomplete which leaves out of consideration a substance which is regularly present in the circulating blood. On the other hand, Howell considers antithrombin to be a very important constituent, ascribing to it the part of maintaining the fluidity of the blood, in that coagulation ensues only when the antithrombin is rendered inert by the neutralizing effect of the zymoplastic substance in the shed blood or in the tissues. As stated, however, there is no diversity of opinion regarding the normal occurrence of antithrombin, so that it would seem worth while to study this substance clinically from a quantitative point of view, in order to obtain fuller data from which to judge its importance. The term antithrombin is employed in a functional sense to designate any substance or substances in the plasma which tend to inhibit coagulation. It is realized that a terminology of this kind cannot be absolutely satisfactory, especially from a chemical point of view. Nevertheless, it appears to be justifiable; it is, for example, in accordance with the physiological studies in immunity, where all the substances,—complement, amboceptor, etc.,—exist only from a functional viewpoint.

There have been very few quantitative examinations of antithrom-

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bin in the human blood. This, probably, may be accounted for by the fact that it has been overshadowed by the striking importance of other substances in the blood,—the thrombin, fibrinogen, and other active principles, which may be grouped as the positive coagulative factors,—and in part by the fact that there has been no simple method for making such tests. The few estimations of this nature which have been carried out have been reported by Howell, who used a method which he has devised. Although this method seems to be satisfactory, it is by no means simple, and is hardly suited to wide clinical use. In the first place, it requires the preparation of a pure thrombin. This is extracted from pig fibrin by treating it with an 8 per cent. solution of sodium chloride, followed by repeated extractions of the coagulable proteins by means of chloroform. The test also requires the preparation of a solution of fibrinogen which must contain no prothrombin; that is, must not clot merely upon the addition of calcium. It requires considerable experience to prepare both of these substances satisfactorily; the fibrinogen solution, which is not the salted fibrinogen of Hammarsten, but a dialyzed plasma, is especially difficult to obtain and to maintain free from contamination of prothrombin.

In the course of testing the coagulability of oxalated plasma from various sources, an examination which included a test for antithrombin, it was found that for clinical use a simple method could be employed, which requires neither a preparation of thrombin nor of fibrinogen. The method is carried out as follows:

About nine cubic centimeters of blood are aspirated and put into one cubic centimeter of 1 per cent. sodium oxalate. The blood is centrifugalized and the plasma removed in the usual way. The plasma is then recalcified by adding 2, 3, 4, and 5 drops, respectively, of a 0.5 per cent. calcium chloride solution. In this way we ascertain the general coagulability of the plasma, which is the composite of a number of interacting factors,—prothrombin, fibrinogen, antithrombin, etc.—and we determine at the same time the optimal amount of calcium for this particular plasma.

If we heat some of this plasma to 60° C., the prothrombin, as is well known, is destroyed and the fibrinogen is coagulated. After filtering off the coagulum we have a plasma which contains anti-

thrombin and practically no prothrombin. The strength of this antithrombin may be ascertained, for clinical purposes, as follows:

First, we prepare human plasma from a normal case just as we prepared the oxalated plasma which is to be tested. Five drops of this plasma are put into five thoroughly cleansed vials. The first of these serves as a control; to the second three drops of normal antithrombin are added; to the third five drops of normal antithrombin; to the fourth three drops of the antithrombin that is to be tested; and to the fifth five drops of this antithrombin. All tubes are equalized in amount by the addition of normal salt solution, and the mixtures are allowed to remain in contact for fifteen minutes. The plasma is then recalcified by the addition of 0.5 per cent. calcium chloride, the number of drops which are added having been determined by the general coagulability test, which should always precede the antithrombin test. As a rule, four drops have been found to be the optimal amount.

TABLE I.  
*Antithrombin Test.*

*A.*

Normal.			Hemophilia.		Interval.
Control.	3 drops antithrombin.	5 drops antithrombin.	3 drops antithrombin.	5 drops antithrombin.	
—	—	—	—	—	2 min.
+	+	+	+	+	4 min.
+++	+++	++	+++	++	6 min.
		+++		+++	8 min.

*B.*

Normal.			Purpura.		Interval.
Control.	3 drops antithrombin.	5 drops antithrombin.	3 drops antithrombin.	5 drops antithrombin.	
—	—	—	—	—	4 min.
+	+	+	+	+	6 min.
+	+	+	+	+	8 min.
+++	+++	++	+	+	10 min.
	+++	++	++	++	12 min.
		++	+++	++	14 min.
		+++		+++	16 min.

Table I *A* illustrates a test of this kind in an atypical case of hemophilia where there was a deficiency of calcium in the blood. We note that antithrombin was present in the plasma of the patient to no

greater degree than in the normal plasma; in both instances coagulation was rapid and but slightly delayed by the addition of three and of five drops of antithrombin. Table I *B* illustrates a similar test in a case of purpura. Here we likewise find no increase in antithrombin.

Antithrombin is judged to be in excess where a marked delay in coagulation is brought about in the tubes to which it has been added, as compared with the coagulation in the control tube. As in the case of the coagulation time, no arbitrary norm can be set up; the results should be well defined to warrant the conclusion that there is an excess of antithrombin.

The essential difference between this antithrombin test and that of Howell is that plasma is used as a basis instead of a fibrinogen solution. The validity of employing plasma in this way may be determined by preparing solutions of hirudin of varying strengths and titrating them upon plasma; in other words, by substituting hirudin for human antithrombin. Tests of this nature were carried out, first upon horse plasma, and later upon normal human plasma. In both cases the plasma was clear and had a coagulation time of not over ten minutes. Table II *A* illustrates an experiment of this kind. We may note that dilutions of 1 to 20,000, 30,000, and 40,000 of hirudin were employed, and that in each instance 1, 3, and 5 drops of one of these antithrombin solutions were added to the human plasma. It will be seen that in each of the three tests the sequence of coagulation is in direct ratio to the number of drops of antithrombin added, and that in general the more dilute the hirudin, the less its inhibiting effect. A test (table II *B*) of normal human antithrombin upon its own plasma has been added to this table to enable a comparison between the strengths of the hirudin used and of human antithrombin. It will be seen that, according to the test, human antithrombin is about equal to a 1 to 40,000 solution of hirudin.

If we turn again to table I, we notice that in addition to the tests of plasma in the case of purpura and of hemophilia, it includes titrations of antithrombin upon autogenous plasma. In these instances the plasma was normal. The same autogenous test may be carried out in pathological cases; *e. g.*, in hemophilia, as shown in table III, which reproduces an equilibrium test of this kind with the

same plasma which is reported in table I. This test evidently can not be considered an antithrombin test, as we employ plasma from a pathological case in order to ascertain the degree of antithrombin. It is termed an equilibrium test because it gives us information as to the balance which obtains in the plasma between all positive and negative factors concerned in coagulation. If the balance is in a state of delicate adjustment, the addition of a small amount of antithrombin will suffice greatly to delay coagulation, whereas if there is an excess of prothrombin and allied substances, this addition will bring about but a slight increase in the coagulation time. We see this when we compare the two tables. In table I, where two equilibrium tests of normal plasma are shown, the addition of three drops of antithrombin resulted in either slight or no delay, and five drops brought about at most a retardation of from ten to sixteen minutes. These tests must not be considered exceptions to the rule, although the delay when three drops of antithrombin are added is generally more marked, and when five drops are added, the period of complete coagulation is postponed to fifteen or twenty minutes. On the other hand, with the plasma of the hemophiliac, referred to in table I, the addition of the same amounts of antithrombin was sufficient to retard coagulation markedly (table III). Comparative tests such as these demonstrate that the mere coagulation time does not furnish complete information as regards the power for clotting which exists in plasma. There are latent potential coagulative factors which come to light only when inhibiting substances are added to the plasma. For example, a normal plasma (table II *A*) coagulated in ten minutes; the plasma of the hemophiliac (table III) in twelve minutes, that is, almost in the same length of time; nevertheless, upon the addition of five drops of its own antithrombin, the former coagulated in sixteen minutes, whereas the latter took forty-three minutes to clot. This test is not recommended for clinical use, because the testing of antithrombin upon abnormal plasma is erroneous. However, it is highly significant from one point of view: it shows that the circulating blood is not delicately balanced in regard to its coagulability. When we reflect that in adding three and five drops of antithrombin to five drops of plasma we are more than doubling its normal content of antithrombin, and that never-

theless coagulation generally ensues with but slight retardation, we must conclude that there exists a considerable factor of safety in the mechanism of the coagulation of the circulating blood. If such were not the case, serious hemorrhage, as the result of a slight temporary excess of antithrombin, would be a constant danger.

TABLE II.

*A. Hirudin.*

Dilution 1:20,000.

1 drop hirudin.	3 drops hirudin.	5 drops hirudin.	Interval.
-	-	-	5 min.
+	+	-	8 min.
++	+	+	10 min.
++	++	+	11 min.
++	++	+	12 min.
+++	++	++	14 min.
	++	++	16 min.
	+++	++	18 min.
		++	20 min.
		+++	22 min.

Dilution 1:30,000.

1 drop hirudin.	3 drops hirudin.	5 drops hirudin.	Interval.
-	-	-	5 min.
+	+	+	8 min.
++	++	+	10 min.
+++	++	++	11 min.
	++	++	12 min.
	++	++	14 min.
	+++	+++	16 min.

Dilution 1:40,000.

1 drop hirudin.	3 drops hirudin.	5 drops hirudin.	Interval.
-	-	-	5 min.
+	+	+	8 min.
++	++	++	10 min.
	++	++	11 min.
	++	++	12 min.
	+++	++	14 min.
		+++	16 min.

*B. Antithrombin Test.*

Control.	1 drop antithrombin.	3 drops antithrombin.	5 drops antithrombin.	Interval.
+	+	+	-	5 min.
++	++	++	+	8 min.
+++	+++	+++	++	10 min.
			++	11 min.
			++	12 min.
			+++	14 min.

TABLE III.  
*Equilibrium Test.*<sup>1</sup>

Control.	3 drops antithrombin.	5 drops antithrombin.	Interval.
—	—	—	6 min.
+	+	+	10 min.
++	+	+	12 min.
+++	+++	+	15 min.
		++	35 min.
		+++	43 min.

#### SUMMARY.

A test is described for the estimation of antithrombin in the blood. The chief advantage of the test is that it is simple, and does not require the preparation of fibrinogen and of thrombin, which are difficult to prepare and to maintain in a pure state. The principle consists in titrating the antithrombin against normal human plasma; in this way we obtain an estimation of its power to delay coagulation. As the result of examinations carried out by this method, it would seem that there is a wide factor of safety as regards the amount of antithrombin in the human blood, and that this inhibiting substance may be increased to a considerable degree without markedly delaying or endangering clotting.

<sup>1</sup> The same case as in table I A.



CONCERNING CHANGES IN THE BIOLOGICAL PROPERTIES OF *TRYPANOSOMA LEWISI* PRODUCED BY EXPERIMENTAL MEANS, WITH ESPECIAL REFERENCE TO VIRULENCE.\*

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During the past few years a great deal of interest has been manifested in biological variations, or mutations, in trypanosomes induced by experimental procedures. That even such simple manipulations as are practiced in maintaining a stock strain of trypanosomes might lead to an appreciable degree of biological alteration in some trypanosomes was rather strongly suggested to me by an instance of exalted virulence in a strain of *Trypanosoma lewisi*, an account of which was published about a year ago.<sup>1</sup>

While others have observed similar examples of virulence in *Trypanosoma lewisi*, notably Jürgens,<sup>2</sup> no satisfactory explanation has been offered for such occurrences. By experimental procedure, however, very marked alterations in the biological properties of this organism have been produced. As early as 1909 Wendelstadt and Fellmer<sup>3</sup> succeeded in producing morphological alterations and an increase in the virulence of *Trypanosoma lewisi* by a series of passages through cold-blooded animals. More recently, the experiments with a reinforced virus, initiated by Roudsky,<sup>4</sup> have demonstrated clearly the possibility of developing a strain of *Trypanosoma lewisi* of a highly virulent character, capable even of serial infection in mice.

Especial importance may be attached to the use of *Trypanosoma lewisi* in such studies on variation, as the relations obtaining between

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<sup>1</sup> Brown, W. H., *Jour. Exper. Med.*, 1914, xix, 406.

<sup>2</sup> Jürgens, *Arch. f. Hyg.*, 1902, xlii, 265.

<sup>3</sup> Wendelstadt, H., and Fellmer, T., *Ztschr. f. Immunitätsforsch., Orig.*, 1909, iii, 422; 1910, v, 337.

<sup>4</sup> Roudsky, D., *Compt. rend. Soc. de biol.*, 1910, lxviii, 421, 458.

host and parasite in the instance of this organism are different from those existing with most laboratory strains of trypanosomes, in that *Trypanosoma lewisi* is one of a few constantly carried in its natural host. While seeking an explanation of our specific problem of virulence, therefore, the attempt was made to approach the problem from a fundamental biological viewpoint with the belief that such information as could be obtained might prove of broader application.

#### SOURCES AND NATURE OF MATERIALS.

In the particular instance of variation that occurred in our stock strain of *Trypanosoma lewisi*, the indications were that either the mode of passage, the character of the rats, or both factors had been instrumental in inducing the change. Accordingly, a simple series of experiments was begun to test the influence of the rate of passage, passage during different periods of the infection, the dose of trypanosomes used, and the character of the rats, as measured by weight, upon succeeding generations of trypanosomes. Two strains of *Trypanosoma lewisi* were used in the tests. One of these, designated as strain I, was the so called pathogenic strain, and the other, strain V, had just been obtained from a natural infection in a young rat. This rat was one of seven infected rats out of a lot of thirty-six obtained from a dealer. The infections in these rats had progressed without symptoms, indicating that the organism was of a relatively harmless type. Very distinct differences between these two strains of *Trypanosoma lewisi* persisted through four parallel series of experiments on white rats. The first series of rats, tables I and II, comprised the stock transfers. These rats were mostly large. They were all inoculated intraperitoneally with two drops of tail blood in 0.5 of a cubic centimeter of salt solution. The rate of passage was necessarily irregular but relatively slow, and in some instances there were several groups of rats inoculated from a common source infection at different periods of the infection, as in generations VI, VII, VIII, and X (table I).

In the second series (tables III and IV) large rats were also used. The inoculations were carried out as in the first series. The transfers were made, however, as near the height of multiplication as pos-

sible, except in the last five generations of strain V; the rate of passage was relatively rapid and uniform.

The third series of experiments, given in tables V and VI, differed from the second only in the use of small rats. The rate of passage in the last seven generations of strain V was intentionally varied here as in series 2.

In the fourth group small rats were used as far as possible (tables VII and VIII), and were inoculated in pairs. At the height of multiplication one animal was killed and its defibrinated blood, up to 0.5 of a cubic centimeter, was injected intraperitoneally into each of the next two rats. Thus the rats in the records do not represent a series of direct transfers but are, as it were, only the controls of the series.

In all these animals accurate records were kept of the course of infections. The blood was examined daily and weights were recorded at least once a week for thirty days. Observations were then discontinued in all except the stock series (series 1) where observations were continued until the termination of the infection.

#### CHANGES PRODUCED IN THE INFECTION CYCLE.

*Incubation Period.*—Other conditions being kept relatively constant, the rate at which *Trypanosoma lewisi* was passed from rat to rat was found to exercise a distinct influence upon the various phases of the infection cycle in succeeding generations of the organism. The earliest evidence of such an effect was seen in the incubation period. An examination of table I shows that of thirty-five rats in only three was the incubation period less than two days, and these rats had been inoculated from infections of seven and ten days' duration. There were six other rats in the series inoculated at relatively the same periods that showed longer periods of incubation. Again, with strain V, table II, we find three rats with incubation periods of one day. Here, however, two of these followed inoculation from an infection of twenty-nine days' duration. Again, there were three rats inoculated from infections of only ten days' duration, or less, with incubation periods of two days. That the tendency of rapid passage was undoubtedly to shorten the incubation period in suc-

TABLE I.  
Slow Passage of Small Doses of Virus in Large Rats.  
*Trypanosoma lewisi*, Strain I, Series I.

Gen- eration.	No.	Weight in gm.	Dy. of passage.	Incubation period in dys.	Trypano- somes in blood.	Symptoms of infection.	Duration of infection in dys.	Termi- na- tion of infection.	Remarks.
I	I	85	5th	5	++	Marked	11	Death	
II	2 * 3	175 132	10th 10th	4 4	++ ++	Marked Marked	11 15	Death Death	
III	4 * 5	116 150	12th 12th	5 4	+++ ++	Marked Marked	8 12	Death Death	
IV	* 6 7 8	146 176 165	8th 8th 8th	3 — —	+++ — —	Marked — —	— — —	Killed — —	Used to test virulence of strain. Killed after 12 dys. Rats 7 and 8 immune.
V	9 *10	70 72	10th 10th	2 2	+++ ++	Marked Moderate	37 107	Killed Recovery	For bacteriological and pathological study. Late symptoms marked.
VI	11 12 13 14 *15	79 162 85 79 127	13th 13th 41st 61st 61st	2 4 5 5 6	++ + +++ ++ ++	Moderate Slight Marked Moderate Slight	— 22 18 14 58	Killed Recovery Death Recovery Recovery	To test virulence of strain. Experiments actually began with this generation.
VII	*16 17 *18	135 93 127	29th 46th 46th	5 — 5	+++ — ++	Marked — Marked	12 — 49	Death — Recovery	Immune. Condition fair.

Gen-eration.	No.	Weight in gm.	Dy. of passage.	Incubation period in dys.	Trypano-somes in blood.	Symptoms of infection.	Duration of infection in dys.	Termination of infection.	Remarks.
VIII	19	114	10th	1	+++	Marked	13	Recovery	Rats 19 and 20 inoculated from 16; others from 18. Rat 19, condition poor.
	20	63	10th	1	+++	Marked	23	Death	
	21	141	12th	2	+++	Moderate	44	Recovery	Condition good.
	22	141	12th	2	+++	Moderate	76	Recovery	Condition good.
	23	143	21st	3	+++	Moderate	34	Recovery	Condition fair.
	24	92	21st	3	+++	Marked	22	Death	
IX	*25	145	32d	3	+++	Marked	35	Death	
	*26	150	7th	1	+++	Slight	61	Recovery	Condition good.
	27	140	18th	—	—	—	—	—	Immune.
	28	146	28th	2	++	Slight	10	Recovery	Condition good.
	29	152	28th	2	+	Slight	10	Recovery	Condition good.
	*30	130	37th	6	+++	Marked	37	Death	
X	*31	126	37th	5	+++	Moderate	130	Death	Late symptoms marked.
	32	110	41st	—	—	—	—	—	Immune.
	33	118	41st	5	+++	Moderate	32	Death	Late symptoms marked.
	34	123	27th	4	+++	Marked	21	Death	Rat 34 inoculated from 30.
	35	119	43d	5	+++	Marked	108	Death	Rat 35 inoculated from 31.

In all the tables where several rats are shown in one generation of transfers, the rats in the direct series are marked with an asterisk (\*).

The figures in the column marked "day of passage" indicate the time elapsing between successive inoculations and refer particularly to the duration of infection in the rat from which a given rat was inoculated. All times estimated are from the time of inoculation.

Relative numbers of trypanosomes in the blood are indicated thus: Very few, +; few, ++; many, +++; great many, +++++.

cessive generations of trypanosomes may be seen by comparing this phase of the infection cycle of the rats in tables I and II with those in the tables following. Still, it must be recognized that the incubation period in any specific instance may be influenced to a great extent by such factors as the age of the infecting organism and the number of the organisms injected.

In this connection a distinction must be drawn between true and false incubation,—a fact that seems to have escaped attention in the literature. Where large numbers of trypanosomes are used to infect an animal, it is not uncommon to find organisms in the blood within a few hours. In the case of the adult forms of *Trypanosoma lewisi* it is easy to show that such an invasion of the blood is not a true incubation, as the type of organism is the same as that injected and the numbers of the organisms subsequently diminish or may entirely disappear from the blood for a day or more before the true invasion of young forms occurs. Where young or multiplying trypanosomes are used, the distinction between true and false incubation becomes arbitrary or fails.

*Periods of Multiplication.*—As with the period of incubation, rapid passage of *Trypanosoma lewisi* tends to advance the time at which multiplication begins in the blood of the rat. In the tabulated records of the rats in series 2, 3, and 4, the figure in the column indicating the "day of passage," in most instances, also indicates the day on which the height of multiplication was reached in the preceding rat. In many of these rats, multiplication was well advanced by the end of the second day after injection and completed by the end of the fourth day. This is in sharp contrast to the usual phase of multiplication, both as to time and duration of the cycle, as well as to the period elapsing between incubation and multiplication. As in the case of incubation, only a few passages were necessary to develop the tendency to shorten the gap between incubation and multiplication, to accentuate the rate of multiplication, and to favor an early termination of the cycle.

*Duration of Infection.*—Infections of *Trypanosoma lewisi* tend to be chronic, to last for thirty days or more, but exceptions to this rule are not at all uncommon; the usual termination is in recovery. In series I of strain V, after the first generation, only one rat out of

TABLE II.  
*Slow Passage of Small Doses of Virus in Large Rats.*  
*Trypanosoma lewisi, Strain V, Series I.*

Gen-eration.	No.	Weight in gm.	Dy. of passage.	Incubation period in dys.	Trypano-somes in blood.	Symptoms of infection.	Duration of infection in dys.	Termination of infection.	Remarks.
I	* 1	117	?	4	++++	Slight	20	Recovery	Condition good.
	2	92	?	5	++++	Slight	22	Recovery	Condition good.
	3	83	?	5	++++	Slight	—	Killed	Killed 8th dy. to test virulence of strain.
II	4	90	4th	2	++++	Slight	86	Recovery	
III	5	90	23d	—	—	—	—	—	Immune.
	* 6	110	39th	3	++++	Moderate	47	Recovery	Condition good.
IV	7	93	34th	4	++++	Moderate	48	Recovery	Condition good.
V	8	153	20th	1	++	Moderate	59	Death	Rats 8 and 9 were infested with lice(?).
	9	145	29th	1	++	Moderate	80	Death	
	*10	167	38th	3	++++	Marked	34	Recovery	
VI	*11	147	15th	2	++++	Marked	14	Death	Immune.
	12	165	18th	—	—	—	—	—	
VII	*13	130	10th	2	++++	Moderate	35	Recovery	Condition fair.
	14	142	10th	2	++++	Moderate	35	Recovery	Condition fair.
VIII	15	154	21st	2	++++	Moderate	27	Recovery	Condition very poor.
IX	16	123	18th	2	++++	Moderate	31	Death	Immune.
	17	114	18th	—	—	—	—	—	
X	18	136	10th	1	++++	Moderate	33	Recovery	Condition poor.
	19	127	29th	2	++++	Marked	45	Recovery	Condition poor.

TABLE III.  
*Rapid Passage of Small Doses of Virus in Large Rats.*  
*Trypanosoma lewisi, Strain I, Series 2.*

Gener- ation.	No.	Weight in gm.	Dy. of passage.	Incubation period in dys.	Trypano- somes in blood.	Symptoms of infec- tion.	Duration of infection in dys.	Termina- tion of infection.	Remarks.
I	1	111	7th	2	+	Moderate	10	Recovery	Virus from rat 11, series 1.
II	2	117	6th	4	+	Marked	10	Recovery	Condition poor.
III	3	135	7th	2	++	Marked	30+	Survival	Condition good.
IV	4	211	4th	2	++	Moderate	30+	Survival	Condition fair.
V	*5	94	4th	1	++	Slight	15	Recovery	Condition fair.
	6	95	4th	1	++	Slight	22	Recovery	Condition fair.
VI	7	102	5th	2	+	Slight	11	Recovery	Condition fair.
VII	8	200	4th	1	++	Slight	30+	Survival	Condition good.
VIII	9	122	3d	2	++	Slight	30+	Survival	Condition good.
IX	10	150	4th	1	+	Marked	6	Recovery	Condition poor.
X	11	114	3d	3	+	Marked	13	Recovery	Condition poor.
XI	12	216	3d	5	++	Marked	12	Recovery	Inoculated from rat 13, series 3. Loss of weight, 46 gm. in 12 dys.



sixteen recovered within the thirty day period of observation (table II). In series 2, 3, and 4, however, where rapid passage was practiced, the last four rats, seven of the last nine, and five of the last six, respectively, recovered within the thirty day period.

In strain I the influence of passage upon the duration of the infection was not so clearly shown and there was only one series of rats, series 2, in which any consistent or definite course of alteration was manifest when compared with series 1. Other factors, which will be considered later, undoubtedly served to mask much of the effect of passage in this strain.

The results obtained with strain V, however, show very clearly what changes may be produced in the several phases of the infection cycle of an ordinary strain of *Trypanosoma lewisi* by the use of only the simplest experimental procedures. Mere regulation of the rate of passage of the virus from one rat to another seems sufficient so to alter the biological properties of *Trypanosoma lewisi* as to change an infection that is usually chronic into one that may be regarded as acute, or *vice versa*. These changes occurred, in my experiments, irrespective of the character of the rats used or of the dose of the infecting organisms.

#### CHANGES IN VIRULENCE.

The basis for estimating the virulence of *Trypanosoma lewisi* in these experiments was the incidence, especially serial incidence, and degree of such symptoms of intoxication as stupor, weakness, loss of weight, and anemia, together with the mortality definitely attributable to infection with *Trypanosoma lewisi*; very little account was taken of isolated instances of severe intoxication or even death.

In series 1 of strain I, the records show that infections with marked symptoms and even death occurred in all except one of the eleven generations of transfers. The virulence of the strain was at its height in the first three generations, though strongly evident again in the last two generations.

In the second series of large rats where the virus was passed rapidly, virulence was much less evident than in series 1. There were, however, well marked symptomatic disturbances in the first

three rats of the series. Even symptoms were then absent until the last two generations when they again became marked. The young rats of the third series showed a larger proportion of severe infections and a few deaths. Crossed infection at the termination of series 2 and 3 showed, however, that there was no appreciable difference in the character of the trypanosomes in the two series at that time (compare rats 11 and 12, series 2, and 13 and 14, series 3). The added influence of large doses of trypanosomes, as in series 4, produced no further alteration in the character of the infection except to insure a consistently shortened incubation period and a greater number of trypanosomes in the peripheral blood. No pronounced virulence was developed by strain V within the limits of these experiments, although the rats of series 1 and 3 showed a gradually increasing number of infections with distinct evidence of intoxication. This was especially evident in the last half of series 3, where the rate of passage was changed with a view to increasing the virulence of the organism. To a less degree, the other series showed a similar increase. The fluctuation in the character of the infections throughout was such as to suggest a cyclic series of changes,—a condition evident also with strain I.

A final factor that appeared to exercise a considerable influence upon the nature of the infection produced by *Trypanosoma lewisi* was the time at which the transfer of the trypanosomes was made. From table I it will be seen that in generation VI three sets of rats were inoculated. Two rats, inoculated from an infection of thirteen days' duration with active agglomeration in progress, developed mild infections. Another rat, inoculated from the same source on the forty-first day of infection, developed a severe infection which terminated fatally, while two other rats inoculated from the same rat on the sixty-first day of infection developed relatively mild infections. A similar condition was observed in generation VIII. Rats 19 and 20, inoculated from rat 16, both showed severe infections. One of these rats recovered after an acute infection and the other succumbed. Rats 21 and 22, inoculated from rat 18 on the twelfth day of infection, developed comparatively mild infections, while the infections in two of three other rats, inoculated from the

TABLE IV.  
*Rapid Passage of Small Doses of Virus in Large Rats.*  
*Trypanosoma lewisi, Strain V, Series 2.*

Gener- ation.	No.	Weight in gm.	Dy. of passage.	Incubation period in dys.	Trypano- somes in blood.	Symptoms of infection.	Duration of infection in dys.	Termination of infection.	Remarks.
I	1	94	4th	2	++	Slight	30+	Survival	Virus from rat 3, series 1.
II	2	92	4th	1	++	Slight	30+	Survival	Condition good.
III	3	90	5th	2	++	Slight	30+	Survival	Condition good.
IV	4	93	4th	1	++	Moderate	30+	Survival	Condition fair.
V	5	140	3d	1	++	Slight	30+	Survival	Condition good.
VI	6	115	4th	1	++	Slight	30+	Survival	Condition good.
VII	7	174	3d	1	++	Slight	30+	Survival	Condition good.
VIII	8	215	3d	2	++	Moderate	30+	Survival	Condition good.
IX	9	210	4th	1	++	Marked	15	Death	Killed after 7 dys. to test virulence of strain.
X	10	217	4th	1	++	Moderate	28	Recovery	Condition fair.
XI	11	145	3d	1	++	Slight	30+	Survival	Condition fair.
XII	12	137	4th	1	++	Marked	18	Death	
XIII	13	132	5th	1	++	Moderate	30+	Survival	Condition fair.
XIV	14	140	6th	2	++	Moderate	18	Recovery	Condition fair.
XV	15	122	8th	2	++	Moderate	14	Recovery	Condition poor.
XVI	16	143	8th	2	++	Moderate	14	Recovery	Condition fair.
XVII	17	110	11th	4	++	Moderate	28	Recovery	Condition poor.

same source on the twenty-first and thirty-second days of infection, terminated fatally. Again, in generation X similar results were obtained.

Similar experiments with strain V were inadequate to warrant independent interpretation, but, as far as they went, they were in harmony with those obtained with strain I.

#### CHANGES IN MORPHOLOGY.

This phase of the subject of biological variation of *Trypanosoma lewisi* has been discussed in detail in another paper.<sup>5</sup> It seems necessary, therefore, only to point out that the variations of morphology observed occurred mainly in infections of considerable severity, indicating a measure of interrelation between virulence and morphological variation.

Before entering upon a discussion of the experiments already described, mention should be made of still another group of experiments, the details of which must be omitted on account of some uncertainty as to their end results. This series of experiments comprised two groups of rats, large and small, with each of the two strains of *Trypanosoma lewisi*.

In these rats the infection was allowed to progress until recovery seemed imminent, when the rat was killed and 0.5 of a cubic centimeter of its defibrinated blood injected into the next rat. With the large rats of both strains and with the small rats of strain I, the trypanosomes were propagated for only a few generations when infection failed, presumably due to the use of immune rats. Two of the series had been interrupted in this manner before it occurred to us to test the immunity of these rats to the stock strain. When infection failed to develop in the third series, after two weeks, this rat was inoculated with the stock strain of *Trypanosoma lewisi* and promptly developed an infection. The possibility of an attenuation by the slow passage of large doses of trypanosomes was neither excluded nor proven, but the most plausible explanation of this result seems to be the transference of sufficient immune bodies with the trypanosomes to protect the new host against infection. This point raises a question of practical importance as to the propriety of a procedure so often employed in attempting to recover trypanosomes

<sup>5</sup> Brown, W. H., *Jour. Exper. Med.*, 1914, xix, 562.

TABLE V.  
*Rapid Passage of Small Doses of Virus in Small Rats.*  
*Trypanosoma lewisi, Strain I, Series 3.*

Gener- ation.	No.	Weight in gm.	Dy. of passage.	Incubation period in dys.	Trypano- somes in blood.	Symptoms of infec- tion.	Duration of infection in dys.	Termi- nation of infection.	Remarks.
I	1	93	7th	2	+	Moderate	10	Recovery	Virus from rat 11, series 1.
II	2	91	6th	5	+	Marked	10	Recovery	Condition poor.
III	3	60	7th	3	++	Marked	30+	Survival	Condition poor.
IV	4	73	4th	2	+++	Marked	—	Killed	Killed after 5 dys. to start series 4.
V	5	70	4th	1	++++	Moderate	30+	Survival	Condition fair.
	*6	68	4th	1	++++	Moderate	30+	Survival	Condition fair.
VI	7	46	5th	1	+++	Marked	26	Death	
VII	8	60	3d	1	++	Moderate	30+	Survival	Condition fair.
	9	60	4th	1	++	Moderate	30+	Survival	Condition fair.
VIII	10	79	3d	2	+	Slight	30+	Survival	Condition good.
IX	11	46	4th	2	++	Marked	20	Death	
X	12	39	3d	1	+++	Slight	22	Recovery	Condition good.
XI	13	51	3d	1	++	Marked	7	Death	
XII	14	63	5th	2	+++	Marked	6	Death	Inoculated from rat 11, series 2.

from an animal suspected of being infected; namely, the transference of large amounts of blood containing but few trypanosomes and an unknown amount of immune bodies. Unfortunately, we have been unable to investigate this subject further.

#### DISCUSSION.

The experiments have necessarily covered a wide field of investigation in order to supply *indications* of the influence of a number of simple factors upon the biological properties of *Trypanosoma lewisi*, and must be so viewed. The conclusions that may be reached on some points are quite clear, while on others there is still some uncertainty.

It is perfectly obvious that, within certain limits, by regulating the time and rate at which the trypanosomes are passed from rat to rat, we may so alter the character of *Trypanosoma lewisi* as to change the course of the infection cycle completely. The development of a strain producing short or acute infections can be accomplished with greater certainty than the intentional reversion of such a strain to a consistently chronic type, or the maintenance of a chronic strain as such; the single factor of rapid passage seems sufficient to accomplish the transformation of a chronic into an acute strain, while in the development or maintenance of a chronic strain the essential conditions are more difficult of control. The occasional short infection in an exceptionally resistant rat, or the occasional severe infection in a susceptible rat, may compel an earlier transfer than would be desirable. Still more difficult to encompass are the cyclic changes in the character of the trypanosomes produced by immunological reactions in the rat's blood during the chronic phase of infection. In a broad sense, the biological status of the trypanosomes at any particular time during an infection may be regarded as only a resultant of these immunological reactions. Since the phenomenon of agglomeration constitutes our only guide to these reactions, the choice of a time when the biological status of the trypanosomes favors our purpose becomes a matter of extreme difficulty.

When we come to consider the question of virulence, this constantly changing status of *Trypanosoma lewisi* in the rat's blood

TABLE VI.  
*Rapid Passage of Small Doses of Virus in Small Rats.*  
*Trypanosoma lewisi, Strain V, Series 3.*

Gen-eration.	No.	Weight in gm.	Dy. of passage.	Incubation period in dys.	Trypano-somes in blood.	Symptoms of infec-tion.	Duration of infection in dys.	Termina-tion of infection.	Remarks.
I	1	55	4th	2	++	Slight	—	Killed	Virus from rat 3, series 1.
II	2	78	4th	1	+	Slight	30+	Survival	Condition good.
III	3	51	5th	2	+++	Marked	10	Death	
IV	4	62	4th	1	+++	Marked	30+	Survival	Condition poor.
V	5	62	3d	1	+++	Marked	30+	Survival	Condition fair.
VI	6	41	4th	1	+++	Moderate	30+	Survival	Condition fair.
VII	7	41	3d	1	+++	Marked	28	Death	
VIII	8	38	7th	1	+++	Marked	27	Death	
IX	9	43	4th	1	+++	Marked	30+	Survival	Condition very poor.
X	10	48	3d	1	+++	Moderate	25	Recovery	Condition good.
XI	11	39	4th	1	+++	Slight	24	Recovery	Condition good.
XII	12	49	5th	1	+++	Moderate	24	Recovery	Condition good.
XIII	13	42	6th	1	+++	Marked	18	Recovery	Condition fair.
XIV	14	38	8th	1	+++	Marked	20	Recovery	Condition fair.
XV	15	54	12th	1	+++	Moderate	21	Recovery	Condition fair.
XVI	16	74	8th	2	+++	Moderate	21	Recovery	Condition good.
XVII	17	63	3d	1	+++	Marked	26	Death	
XVIII	18	65	18th	2	+++	Marked	7	Death	

assumes even greater importance. Apparently the conditions to be met in maintaining or building up a virulent strain of *Trypanosoma lewisi* are indicated by the conditions obtaining in severe infections, of which I have observed three main types.

In one small group of such infections large numbers of trypanosomes persist in the blood with but scant evidence of their destruction. These infections are chronic and differ from the usual infection only in degree.

In the second and largest group the number of trypanosomes is never great, and degeneration and phagocytosis of trypanosomes are comparatively prominent. These infections may terminate very early,—usually in recovery,—or when multiplication persists beyond the accustomed limits the infection is more prolonged and not infrequently terminates fatally.

In the third and most important group multiplication is extremely active and irregular in character; the blood swarms with trypanosomes, and degeneration and disintegration of trypanosomes are marked; there is a marked leucocytosis, and the blood contains a large number of active phagocytes. Death of the rat usually occurs while multiplication is still active; recovery is exceptional.

If the last group be taken as the highest development of the virulent type, three factors in the infection become significant: vigorous reproduction, limited vitality of the trypanosomes, and a strong defensive (?) response on the part of the host. The association of vigorous reproduction with weakened resistance to destruction may appear somewhat paradoxical. There appears to be some distinction, however, between the mechanism limiting multiplication and that causing the destruction of trypanosomes in the rat's blood. In the usual infection of *Trypanosoma lewisi* we have evidence of what I must regard as two distinct classes of immunological reaction, one of which is concerned in checking the multiplication of trypanosomes, as ordinarily understood, and the other in their destruction. That these phenomena are in reality separable is shown by the fact that in some infections multiplication in the peripheral blood may be completely checked early in the infection with no appreciable decrease in the numbers of trypanosomes or other evidence of their destruction for weeks or even months. On the other hand, in certain infections, as indicated above, active multiplication may



TABLE VII.  
*Rapid Passage of Large Doses of Virus in Small Rats.*  
*Trypanosoma lewisi, Strain I, Series 4.*

Gener- ation.	No.	Weight in gm.	Dy. of passage.	Incubation period in dys.	Trypano- somes in blood.	Symptoms of infec- tion.	Duration of infection in dys.	Termina- tion of infection.	Remarks.
I	1	74	5th	1	++	Slight	30 +	Survival	Virus from rat 4, series 3.
II	2	102	4th	1	+++	Slight	30 +	Survival	
III	3	57	2d	1	+++	Marked	30 +	Survival	Condition very poor.
IV	4	200	2d	1	+++	Marked	17	Death	
V	5	62	3d	1	+++	Slight	30 +	Survival	Condition good.
VI	6	58	2d	1	+++	Slight	30 +	Survival	Condition fair.
VII	7	67	2d	1	+++	Moderate	25	Recovery	Condition good.
VIII	8	63	3d	1	+++	Slight	30 +	Survival	Condition good.
IX	9	48	2d	1	+++	Slight	30 +	Survival	Condition good.
X	10	70	3d	1	+++	Moderate	30 +	Survival	Condition poor.
XI	11	61	3d	1	+++	Marked	6	Death	
XII	12	53	3d	1	+++	Slight	25	Recovery	Condition fair.
XIII	13	48	3d	1	+++	Slight	30 +	Survival	Condition fair.
XIV	14	64	4th	1	+++	Moderate	30 +	Survival	Condition fair.
	15	56	4th	1	+++	Moderate	30 +	Survival	Condition fair.

continue beyond its usual limits, while the number of trypanosomes in the peripheral blood continually diminishes with abundant evidence of vigorous destruction.

The essential elements of a spontaneously acquired virulence of *Trypanosoma lewisi* appear, therefore, to be closely related to immunological reactions and to be dependent upon an appreciable degree of reproductive fastness, strong antigenic properties, and weakened resistance to destruction. Such an hypothetical basis for a naturally acquired virulence or for a virulence developed in *Trypanosoma lewisi* as the result of a system of passage obviously constitutes a state of unstable balance of the biological properties of the organism. It is not inconceivable, however, that such a condition might be maintained or developed by a fortunate system of passage that takes due account of the existing biological status, or balance, of the organism to be dealt with. For example, strain V of my experimental series was a normally balanced strain and showed a consistent response to the experimental procedures employed. Strain I, on the contrary, was highly unstable so that under the same experimental conditions as strain V its fluctuations were frequent and sharp with but little persistent tendency to change in any given direction.

Moreover, reference should be made to the animal equation in experimental modification of *Trypanosoma lewisi*. As far as resistance to infection is concerned, small rats seemed to offer less resistance or were more susceptible to intoxication than large rats, as others have repeatedly observed. Theoretically, it should be possible to utilize this factor, since in its final aspects the problem is one of host and parasite, with the host supplying the mechanism through which the desired result may be accomplished; it is this feature of the reaction with which we are least able to reckon.

The extent of these experiments does not permit of deductions as to the permanency of any of the acquired properties of *Trypanosoma lewisi* that have been discussed. The tendency to revert to the natural or stable form of balance was quite evident in all instances as far as my experiments went, but much more extended series would be necessary before an opinion could be expressed as to the impress left upon *Trypanosoma lewisi* by such experimental procedures.

TABLE VIII.  
*Rapid Passage of Large Doses of Virus in Small Rats.*  
*Trypanosoma lewisi, Strain V, Series 4.*

Generation.	No.	Weight in gm.	Dy. of passage.	Incubation period in dys.	Trypanosomes in blood.	Symptoms of infection.	Duration of infection in dys.	Termination of infection.	Remarks.
I	1	73	5th	1	++++	Slight	30+	Survival	Series started from rat 1, series 3. Condition good. Infection very light and condition poor at 30 dys.
II	2	96	4th	1	++++	Slight	30+	Survival	
III	3	51	2d	1	++++	Moderate	30+	Survival	
IV	4	200	2d	1	++++	Marked	22	Death	Condition good. Condition fair. Condition poor. Condition poor. Condition good. Condition good. Condition good.
V	5	75	1st	1	++++	Slight	30+	Survival	
VI	6	62	2d	1	++++	Moderate	30+	Survival	
VII	7	54	2d	1	++++	Moderate	30+	Survival	Condition poor. Condition good. Condition good.
VIII	8	92	2d	1	++++	Slight	30+	Survival	
IX	9	72	2d	1	++++	Slight	30+	Survival	
X	10	68	2d	1	++++	Slight	30+	Survival	Condition good. Condition fair. Condition poor.
XI	11	63	3d	1	++++	Slight	30	Recovery	
XII	12	56	3d	1	++++	Moderate	22	Recovery	
XIII	13	47	3d	1	++++	Moderate	28	Recovery	Late symptoms marked. Condition very poor. Condition good. Condition good.
XIV	14	38	3d	1	++++	Marked	30+	Survival	
XV	15	53	4th	1	++++	Moderate	29	Recovery	
	16	61	4th	1	++++	Slight	18	Recovery	Condition good.

In conclusion, since the object of the investigation was to obtain indications of the nature and mode of action of the factors influencing the biological properties of *Trypanosoma lewisi*, the results must be interpreted in a broad sense, as much of the detail requires long and careful study. The experiments have served, however, to give a clearer insight into the relations existing between *Trypanosoma lewisi* and its host and afford a basis for formulating a conception of the essential elements of the virulence of this organism. The results also indicate that immunological reactions of two distinct types exercise a dominant influence as determinative factors in natural and experimental modifications of the species, and that by judicious control of these forces marked changes in the biological properties of *Trypanosoma lewisi* may be accomplished.

#### SUMMARY.

1. Different strains of *Trypanosoma lewisi* represent different states of biological balance, especially between the powers of propagation and resistance to destruction.
2. The biological status of a given strain of *Trypanosoma lewisi* is subject to cyclic variations as the result of immunological reactions in the blood of the host.
3. The factors limiting reproduction and causing destruction of *Trypanosoma lewisi* in the blood are appreciably independent of each other. It is possible, therefore, to influence these processes separately and even in opposite directions.
4. The virulence of *Trypanosoma lewisi*, manifested in its highest form, is dependent upon some degree of reproductive fastness, strong antigenic action, and susceptibility to destruction, varying degrees in the development of these properties producing corresponding variations in the degree of virulence.
5. By a properly regulated system of passage the properties of *Trypanosoma lewisi* that determine its infection cycle and its virulence may be eventually so altered as to change completely both the nature and course of the infection. Such a system of passage must be adapted to the particular strain of *Trypanosoma lewisi* used.
6. Immunological reactions exercise a dominant influence in determining the ultimate biological variations of *Trypanosoma lewisi*.

# THE INFLUENCE OF MILK FEEDING ON MORTALITY AND GROWTH, AND ON THE CHARACTER OF THE INTESTINAL FLORA.\*

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## I. THE INFLUENCE OF MILK FEEDING ON MORTALITY AND GROWTH.<sup>1</sup>

The investigations which furnish the data presented in this paper extended over a period of about three years. The milk feeding experiments were the direct outcome of the researches that had been conducted for several years at the Storrs Agricultural Experiment Station on bacillary white diarrhea of chicks, and had as their chief object a study of the value of sour milk as a possible preventive and even curative agent in the elimination of bacillary white diarrhea. It was anticipated, in conformity with the views of Metchnikoff and his followers, that sour milk, in virtue of the acids that it contains, or as the immediate result of the acid-producing bacteria, might exert a beneficial influence in preventing or allaying the disease, if supplied soon enough.

The results of the first year were such as to leave little doubt as to the life-saving value of sour milk, in as far as bacillary white diarrhea was concerned. What appeared to be of far greater significance, however, was the marked influence of sour milk on the growth of the chicks and on the total death rate or mortality from all causes. In subsequent feeding experiments milk that was soured by *Bacillus bulgaricus* and sweet milk were employed, as well as ordinary sour

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<sup>1</sup> For references on the subjects of diet and growth, the reader is referred to the publications of Osborne and Mendel (Osborne, T. B., and Mendel, L. B., *Carnegie Institution of Washington Publications*, 1911, No. 156. Osborne, T. B., Mendel, L. B., and Ferry, E. L., *Ztschr. f. physiol. Chem.*, 1912, lxxx, 307; *Jour. Biol. Chem.*, 1912, xii, 81, 473; 1912-13, xiii, 233. Osborne, T. B., Mendel, L. B., Ferry, E. L., and Wakeman, A. J., *Jour. Biol. Chem.*, 1913, xv, 311; 1913-14, xvi, 423; 1914, xvii, 325, 401).

milk. Throughout the different series of experiments<sup>2</sup> the plan of investigation was essentially the same, and but for certain minor details was as follows:

The chicks, with few exceptions, were hatched in artificial incubators under as nearly uniform conditions as possible. When taken from the incubators the apparently sound chicks were divided uniformly into different lots, usually six. The number in each lot varied in the different experiments from fifteen to sixty. When sour milk was the only milk that was fed, as in the first series, the different lots or pens received the following treatment: Pen 1 was artificially infected with a bouillon culture of *Bacterium pullorum* when 24 to 36 hours old, and was fed sour milk; pen 2 was similarly infected, but received no milk; pen 3 was infected when 48 to 60 hours old and was fed sour milk; pen 4 was infected at the same age as pen 3, but was given no milk; pen 5 served as an uninfected control with sour milk, and pen 6 as an uninfected control without the milk.

In the numerous experiments which involved the use of two kinds of milk, as for example sweet and ordinary sour milk, the chicks were divided into equal lots, usually six. Pens 1, 2, and 3 were artificially infected with *Bacterium pullorum*, while pens 4, 5, and 6 were left uninfected. Pens 1 and 4 were fed the ordinary sour milk; pens 2 and 5 received the sweet or *bulgaricus* milk, according to the prearranged plan, and pens 3 and 6 went without milk. Thus double sets of controls were provided for.

Artificial infection with *Bacterium pullorum*, the organism which causes bacillary white diarrhea in chicks, was brought about by the use of a forty-eight hour bouillon culture which was administered with the aid of a medicine dropper. Three to six drops of the culture were placed in the beak in such a manner that the chick was compelled to swallow them. The milk was supplied in shallow galvanized pans having a capacity of about one pint. In order to prevent the chicks from wading in the pans coarse mesh wire was fastened over the tops. The pans were thoroughly cleaned at least

<sup>2</sup> Rettger, L. F., Kirkpatrick, W. F., and Stoneburn, F. H., *Bull. Storrs Agricultural Experiment Station*, 1912, No. 74. Rettger, L. F., Kirkpatrick, W. F., and Jones, R. E., *ibid.*, 1914, No. 77. Rettger, L. F., Kirkpatrick, W. F., and Card, L. E., *ibid.*, 1915, No. 80.

once a day, and scalded at definite intervals. In most of the experiments the milk was supplied twice a day.

Ordinary sour milk was usually obtained by adding a starter (sour milk) to fresh skimmed milk the day before it was to be used. The milk was kept at ordinary summer room temperature (25°–30° C.) either in pails or in milk bottles. *Bulgaricus* milk was prepared by sterilizing fresh skimmed milk, and after sufficient cooling, inoculating it with pure milk cultures of *Bacillus bulgaricus* which was procured in powder or tablet form from two reliable sources. The acidity was determined from day to day by titration. As a rule, the acidity was not permitted greatly to exceed 1.0 per cent. (in terms of lactic acid), since less of the milk was consumed when the acidity was high. The *bulgaricus* milk had the appearance of the product that is usually obtained with the use of *Bacillus bulgaricus*. Junket tablets were added in all but two instances to the sweet milk, in order to make it appear more attractive to the chicks. The conclusion was finally arrived at, however, that the junket tablets were unnecessary.

All the chicks were constantly supplied with an excess of feed. This consisted of the ordinary chick feed during the first week, after which a standard dry mash was fed. After the first year the feed going into each pen was weighed, and the amount left in the pans again weighed at the end of definite periods. The milk was also weighed, and complete records were kept of the milk and feed consumed per week.

The chicks were weighed at the beginning of the experiments, and once a week at as nearly the same hour as was possible. Daily mortality records were kept, and postmortem examinations were made of the chicks that died during the course of the experiments. Special emphasis was placed on the recovery of *Bacterium pullorum* from the internal organs and from the unabsorbed yolk.

In the entire investigation 5,118 chicks were employed. The majority of them were white Leghorns, though all the experiments of 1913 were conducted on white Plymouth Rocks. The small number of Rhode Island Red chicks that was used was of comparatively low vigor and was, therefore, less satisfactory than the others. The chicks were housed in small outdoor brooder houses which opened into large yards or runs, and in a large brooder house

from which long runs extended from each pen. About the same number of experiments was conducted under the two sets of housing conditions. The yards to which the chicks had access contained sufficient grass to supply them with the necessary amount of green food. The experiments were continued until the chicks were six to seven weeks old, when they were removed to permanent quarters.

#### RESULTS OF THE MILK FEEDING EXPERIMENTS.

Since no records of the amounts of feed and of sour milk consumed were kept in the investigations of the first year, the results are of far less importance than those of the two following years. Furthermore, the lack of complete uniformity in the plans of the different experiments makes the averaging of results somewhat difficult and unsatisfactory. It was most clearly shown throughout the work, however, that the feeding of sour milk exerted a beneficial influence on the growth and mortality of the chicks. The following tabulated results (table I) of experiment F will serve as a good illustration.

TABLE I.

	Infected when 36 hrs. old.		Infected when 60 hrs. old.		Uninfected controls.	
	Pen 1. Fed sour milk.	Pen 2. No milk.	Pen 3. Fed sour milk.	Pen 4. No milk.	Pen 5. Fed sour milk.	Pen 6. No milk.
Weight per 10 chicks.....	5.45 lbs.	3.07 lbs.	4.75 lbs.	3.22 lbs.	5.64 lbs.	3.14 lbs.
Mortality.....	3 or 8.1%	8 or 21.6%	2 or 5.4%	6 or 16.2%	0	4 or 10.8%

The total number of chicks used in the experiment was 222, or 37 in each pen.

In several instances the weight of the milk-fed chicks was, at the end of the experiment, at least double that of the chicks that received no milk, whether they were infected with *B. pullorum* or not. The sour milk chicks were stronger and more vigorous than the others, particularly in the uninfected pens. This was shown in different ways, as for example in the size and color of the combs, the strength of the feet and legs, and in the various activities of the chicks.

The sour milk feeding exerted a decided influence on mortality from bacillary white diarrhea as well as from all causes. In all of five complete experiments the mortality of the infected chicks was lower in the pens that were supplied with milk than in those which were not. In several instances the number of deaths in the lots with no milk was at least twice as great as in the corresponding pens that received milk, and in one case the ratio was approximately 3:1. Since the investigation of this first year involved the use of 1,044 chicks, some importance should be given to these results. In two experiments that were successfully carried through, and in which there were uninfected control lots, it happened that not a single death occurred in the sour milk pens that were not artificially infected with *B. pullorum*, while in the corresponding lots with no milk the mortality was approximately 11 per cent.

The results of the first year are in perfect harmony with those of subsequent investigations, in so far as the influence of milk feeding on growth and general



mortality is concerned. The apparent discrepancies that exist in the data of 1912 and 1913, bearing on the influence of milk feeding on the mortality from white diarrhea, may be fully explained, as will be seen later.

As none but ordinary sour milk was employed in the above experiments, no satisfactory explanation presented itself as to how such significant results of a sour milk diet could be brought about. It seemed to be of considerable importance, therefore, to determine, if possible, to what factor or factors milk owed these properties. It was for this purpose largely that the extensive investigations of the following two years were carried on. More complete data were sought, also, on the value of milk feeding as such.

In the second series of investigations seven different experiments were carried on and successfully completed. In three of them ordinary sour milk was the only milk that was supplied, while in the remaining four both the naturally soured and *bulgaricus* milk were used. Altogether 1,824 chicks were employed. In the first three experiments full records were made of the total gain in weight per ten chicks, the total feed consumed, the gain in weight per lb. of feed, and the mortality. In three of the four experiments in which a comparative study was made of ordinary sour and of *bulgaricus* milk the following data were acquired: total gain per ten chicks, total feed consumed, total milk consumed, total solid matter fed including the milk, per cent. acid in milk, gain per ten chicks for each lb. of solid food, and mortality. As the fourth experiment was conducted on hen-hatched and hen-reared chicks, no food records could be kept.

Time and space do not permit of a complete survey of the results here;<sup>3</sup> hence only brief summaries will be given.

TABLE II.

*The Influence of Sour Milk Feeding on the Mortality of Infected and Uninfected Chicks.*

Experiment.	No. of chicks in each pen.	Total mortality.			
		Infected chicks.		Uninfected chicks.	
		Fed sour milk.	No milk.	Fed sour milk.	No milk.
A	37	23	20	17	27
	37	23	20		
B	54	16	12	1	17
	54	7	13		
C	50	29	39	2	12
	50	18	16		
D	50	7	8	0	31
E	15	3	4	0	0
F	53	18	27	6	8
G	45	25	28	11	16
Total <sup>4</sup>		169, or 38%	187, or 42%	37, or 12.2%	111, or 36.5%

<sup>3</sup> For detailed statements of the results, including tables and curves, the reader is referred to Rettger, Kirkpatrick, and Jones, *loc. cit.*

<sup>4</sup> 445 in each of the two infected groups, and 304 in each of the two uninfected groups.

While the difference in the mortality of the two general infected groups (one supplied with sour milk and the other not) is but 4 per cent., the influence of sour milk feeding on mortality from all causes is encouraging (table II). Of the 304 uninfected chicks that were supplied with sour milk 37, or 12.2 per cent., died, as compared with a mortality of 36.5 per cent. in the corresponding group that received no milk. In other words, there were only one-third as many deaths in the former as in the latter. The mortality as a whole in the uninfected lots is considerably greater than it was during the previous year. This is undoubtedly due to a difference in the vitality of the chicks. In the investigations of 1912 strong white Leghorn stock was usually employed, while in the subsequent year white Plymouth Rock chicks which possessed no unusual vigor served as the subjects of investigation.

In the investigations of 1913 the chicks which were artificially infected with bouillon cultures of *B. pullorum* were subjected to the infecting process only once; that is, aside from the possibilities of natural infection between chicks, the chicks received but one treatment with the organism, and that was at practically the same time that the sour milk was supplied.<sup>5</sup>

TABLE III.

*Combined Data Showing the Influence of Sour Milk Feeding on Growth.*

	No. of chicks in experiments,	Gain per ten chicks.		
		Fed sour milk.	No milk.	Difference.
Infected lots. . . .	890	7.0 lbs.	4.3 lbs.	3.7 lbs., or 38.5%
Uninfected lots. . .	608	7.9 lbs.	4.6 lbs.	3.3 lbs., or 41.8%

The above summaries (table III) do not include any data on the feeding of *bulgaricus* milk. They are based on work that was in a large measure merely a continuation of the investigation of the previous year. In the following condensed statements the results of the feeding of milk that was soured by *B. bulgaricus* are included, and will therefore serve to show the relative merits of the two methods (table IV).

TABLE IV.

*Gain Per Ten Chicks for Each Pound of Solid Matter, Including Milk Solids, Consumed.*

	No. of chicks in experiments.	Gain per lb. of solid matter consumed.		
		Fed sour milk.	Fed <i>bulgaricus</i> milk.	No milk.
Infected lots. . . .	444	0.29 lb.	0.30 lb.	0.29 lb.
Uninfected lots. . .	444	0.32 lb.	0.30 lb.	0.26 lb.
Combined average	888	0.305 lb.	0.30 lb.	0.28 lb.

<sup>5</sup> The term sour milk is used throughout this paper to designate ordinary or naturally soured milk, in distinction from *bulgaricus* milk.

It is of interest to note that in the sour milk and the *bulgaricus* milk groups the gains per lb. of solid matter consumed are practically the same. In the lots that were not supplied with milk the average gain was noticeably less (approximately 8 per cent.). This difference, when taken by itself, is too small to be of much significance, but when considered along with other data should have some importance.

In order to make a comparative study of sour and of *bulgaricus* milk with special emphasis on acidity as an important factor, it was necessary to keep a full record of the acidity of both the sour and the *bulgaricus* product, and of the amounts fed. In table V the acidity is given in terms of lactic acid.

TABLE V.  
*Amounts of Acids in Milk Consumed.*

Experiment.	Infected chicks.		Uninfected chicks.	
	Fed sour milk.	Fed <i>bulgaricus</i> milk.	Fed sour milk.	Fed <i>bulgaricus</i> milk.
D	0.19 lb.	0.45 lb.	0.21 lb.	0.44 lb.
F	0.12 lb.	0.15 lb.	0.18 lb.	0.20 lb.
G	0.12 lb.	0.12 lb.	0.16 lb.	0.17 lb.
Total	0.43 lb.	0.72 lb.	0.55 lb.	0.81 lb.

The total amount of ordinary sour milk consumed was 115.6 lbs., and that of *bulgaricus* milk 105.5 lbs. In spite of this difference the total amount of acids in the *bulgaricus* milk (1.53 lbs.) was far in excess of what the sour milk contained (0.98 lb.). If the value of sour milk feeding is dependent upon the amounts of acids, or the degree of acidity, present, the results obtained with the *bulgaricus* milk should have been far better than those following the sour milk diet. That this was not the case is clearly shown in the following condensed results (table VI).

TABLE VI.  
*Summary Showing the Comparative Influence of Sour Milk and of Bulgaricus Milk on Mortality.*

Experiment.	No. of chicks.	Mortality.			
		Infected chicks		Uninfected chicks.	
		Fed sour milk.	Fed <i>bulgaricus</i> milk.	Fed sour milk.	Fed <i>bulgaricus</i> milk.
D	50	7, or 14%	10, or 20%	0	6, or 12%
F	53	18, or 34%	20, or 37.7%	6, or 11.3%	8, or 15.1%
G	45	25, or 55.5%	18, or 40%	11, or 24.4%	8, or 17.7%
Total	148	50, or 33.7%	48, or 32.4%	17, or 11.5%	22, or 14.8%

These figures require little comment. The total mortality of all chicks that received the naturally soured milk (both infected and uninfected) was 67, or 45.3 per cent., as compared with 70, or 47.3 per cent., for the *bulgaricus* chicks.

The small difference which is to the advantage of the sour milk feeding is, of course, within the limits of possible error in experiments of this kind, and should not be given much importance; nevertheless, the results clearly show that *bulgaricus* milk is of no greater value in reducing mortality than ordinary sour milk.

TABLE VII.

*Figures Showing the Comparative Influence of Sour Milk and of Bulgaricus Milk on Growth (Experiments D, E, F, and G).*

*Average Gain per Ten Chicks.*

Infected chicks.		Uninfected chicks.	
Fed sour milk.	Fed <i>bulgaricus</i> milk.	Fed sour milk.	Fed <i>bulgaricus</i> milk.
6.13 lbs.	5.61 lbs.	7.50 lbs.	6.19 lbs.

Combined averages for infected and uninfected chicks:

Fed sour milk .....	6.82 lbs.
Fed <i>bulgaricus</i> milk .....	5.90 lbs.
Difference .....	0.92 lb.

These figures (table VII) are for experiments D, E, F, and G. If we eliminate experiment E because hens were used as brooders and no records could be made of the amounts of feed and milk consumed, the averages are as follows (table VIII).

TABLE VIII.

*Average Gain per Ten Chicks in Experiments D, F, and G.*

Infected chicks.		Uninfected chicks.	
Fed sour milk.	Fed <i>bulgaricus</i> milk.	Fed sour milk.	Fed <i>bulgaricus</i> milk.
5.48 lbs.	4.87 lbs.	7.1 lbs.	5.87 lbs.

Combined averages for infected and uninfected chicks:

Fed sour milk .....	6.26 lbs.
Fed <i>bulgaricus</i> milk .....	5.37 lbs.
Difference .....	0.89 lb.

The pens that were supplied with the naturally soured milk (experiments D, F, and G) gained 0.89 of a lb. (or 14.2 per cent.) more per ten chicks than those which received the *bulgaricus* product. Since the former consumed more food (both dry feed and milk), but less acid, than the *bulgaricus* group, the greater gain in the sour milk pens must be attributed almost entirely to the food as such. The difference in gain per ten chicks closely corresponds with the difference in the total amount of solid food taken, namely 12.9 per cent., the sour milk chicks consuming 20.45 lbs. and the *bulgaricus* lots 17.81 lbs. Here again the differ-

ence is in favor of the naturally soured milk. Not only was there a larger absolute gain in weight in the sour milk chicks, but the gain per lb. of solid matter consumed was greater than in the *bulgaricus* lots. This difference has already been brought out in table II. The sour milk was the more appetizing, as 115.6 lbs. were consumed in experiments D, F, and G, while only 105.5 lbs. of the *bulgaricus* milk were utilized.

#### A COMPARATIVE STUDY OF THE INFLUENCE OF SWEET AND OF SOUR MILK ON MORTALITY AND GROWTH.

In the investigation of the past year (1914) eight complete experiments involving the use of 2,250 chicks were conducted.<sup>6</sup> The plan of these experiments was practically the same as the preceding.

Each lot of newly hatched chicks was divided into six uniform groups. Pens 1, 2, and 3 were artificially infected with 48 hour bouillon cultures of *B. pullorum*, while pens 4, 5, and 6 were left untreated. Pens 1 and 4 were fed ordinary well soured milk; pens 2 and 5 sweet milk, and pens 3 and 6 no milk at all. Thus, pens 4, 5, and 6 served as controls for infected pens 1, 2, and 3; and pens 3 and 6 were controls with no milk for pens 1, 2, 4, and 5. In all but two of the experiments the sweet milk was curdled with rennet tablets. The milk was supplied at least twice each day, usually early in the morning and at noon. In every case of milk feeding the milk was supplied as soon as the chicks were removed from the incubators, approximately twenty-four hours after hatching. In six of the experiments the chicks were artificially infected at the time that they first received the milk, as well as on each of the following four days. In the other two experiments the first infection was postponed until three or four days after the earliest milk feeding. In every instance the bouillon culture was administered five times on five consecutive days. The delaying of infection with *B. pullorum* was for the purpose of giving the chicks all the advantages of early milk feeding that it might possess, especially in as far as increasing bodily vigor is concerned.

The results of these experiments appear in the tables<sup>7</sup> under the following heads: numbers of chicks in each pen at the beginning of each week, weekly weights of the chicks, amounts of dry feed and of milk consumed per week as well as for the entire period, gains in weight per ten chicks for each pound of total solid matter, including milk consumed, and the mortality. The milk solids were estimated as 10 per cent. Table IX is a condensed statement of the data which bear on mortality.

<sup>6</sup> Rettger, Kirkpatrick, and Card, *loc. cit.*

<sup>7</sup> Rettger, Kirkpatrick, and Card, *loc. cit.*

TABLE IX.

*Influence of Sweet and of Sour Milk Feeding on Mortality.*

Experiment.	No. of chicks.	Mortality.					
		Infected chicks.			Uninfected chicks.		
		Fed sour milk.	Fed sweet milk.	No milk.	Fed sour milk.	Fed sweet milk.	No milk.
A	31	11	4	4	10	4	5
B	38	17	22	31	9	10	2
C	38	20	22	24	4	3	5
D	59	2	4	10	3	6	3
E	49	30	30	40	3	10	25
F	60	49	48	60	25	23	50
G	50	16	9	24	5	2	10
H	50	6	6	8	6	3	14

Total mortality, by pens    151,        145,        201,        65,        61,        114,  
    or 40%    or 39%    or 54%    or 17%    or 16%    or 30%

Total mortality for the three infected groups.....497, or 44.2 per cent.

Total mortality for the three uninfected groups...240, or 21.3 per cent.

The results obtained in experiments A and B were not as satisfactory and well defined as in the others. The chicks were hatched in March, and hence were kept indoors for the greater part of the time that they were under observation, the weather being cold. Considerable leg weakness developed, which was confined largely to the pens receiving milk. The sour milk pens in A were most seriously affected. More milk was consumed by these chicks than in the sweet milk lots. Furthermore, the conditions were more favorable for leg weakness at the time experiment A was in progress than later. It appeared most evident that the milk-fed chicks were growing too fast, under the conditions of close confinement, for the legs to support their weight.

The mortality in experiment F was unusually high in all the pens, especially in the uninfected lots. These figures alone constitute a large part of the total mortality. They are explained by the fact that the chicks used in this experiment were of low vitality, which was shown in various ways, aside from the high death rate. However, the proportion that the mortality figures of the milk-fed chicks bear to those which were not supplied with milk is about the same as the average for the eight experiments.

According to the above data there is a slight difference in the value of sweet and of sour milk feeding. Among the infected chicks the mortality was 2.5 per cent. greater in the sour milk than in the sweet milk pens, while among the uninfected the difference amounted to 5.9 per cent.

The value of milk feeding as such is clearly demonstrated in the

table. Not only was there a great reduction in the mortality of the uninfected lots that received milk (either sweet or sour), which amounted to almost 100 per cent., but there was a marked difference in the death rate of the milk-fed infected chicks, as compared with those which remained without milk, the difference amounting to approximately 30 per cent. In other words, there were almost twice as many deaths in the uninfected lots which were denied the milk than in the corresponding sweet or sour milk pens, and almost one-third more deaths in the infected groups that did not receive the milk than in the corresponding milk-fed lots. These results are in harmony with those of the first series of experiments (1912). Sufficient evidence is at hand, therefore, to show that the feeding of milk, either sweet or sour, exerts a most decided influence in lowering the general death rate of young chicks, and if fed soon enough greatly reduces the mortality from bacillary white diarrhea.

That the feeding of sour milk in the second series of investigations did not materially affect the death rate from bacillary white diarrhea may be explained readily by the fact that the milk was not supplied soon enough before the chicks were subjected to the artificial infection, and hence failed to increase the vigor and resistance of the chicks before the bacteria in question established themselves and the disease ran its natural course.

TABLE X.

*Influence of Sweet and of Sour Milk Feeding on Growth.  
Average Total Gain in Weight per Ten Chicks.*

Infected chicks.			Uninfected chicks.		
Fed sour milk.	Fed sweet milk.	No milk.	Fed sour milk.	Fed sweet milk.	No milk.
4.62 lbs.	4.17 lbs.	2.65 lbs.	5.07 lbs.	4.71 lbs.	3.12 lbs.

Combined averages, all chicks:

Fed sour milk .....	4.84 lbs.
Fed sweet milk .....	4.44 lbs.
Fed no milk .....	2.88 lbs.

TABLE X.—*Concluded.*

*Gain in Weight per Ten Chicks for Each Pound of Total Solid Matter Consumed as Food.*

	No. of chicks employed.	Fed sour milk.	Fed sweet milk.	No milk.
Infected.....	1,125	0.25 lb.	0.24 lb.	0.19 lb.
Uninfected.....	1,125	0.27 lb.	0.26 lb.	0.22 lb.
Combined (infected and uninfected).....	2,250	0.26 lb.	0.25 lb.	0.205 lb.

In every instance (table X) the feeding of sweet and of sour milk was followed by a marked increase in the weights of the chicks beyond that which took place in the chicks which received no milk, the difference often amounting to as much as 80 per cent., and in two cases to more than 100 per cent. The differences vary in a large measure in direct proportion to the amounts of total solid matter consumed. Nevertheless, a review of the original tables<sup>8</sup> will show that, aside from its appetite-stimulating properties, the milk served to bring about a more complete utilization of the food.

The combined data on the 2,250 chicks show that those which received the sour milk gained 0.26 of a pound per ten chicks for each pound of total solids consumed; that those which were fed sweet milk made a gain of 0.25 of a pound; and those that were without milk gained only 0.20 of a pound. In other words, the milk-fed chicks gained 25 per cent. and 30 per cent. more in weight per pound of solid matter used than the chicks which received no milk. The gains per pound of solid food in the sour milk chicks were 5 per cent. greater than in the sweet milk lots. As this difference is so small as to lie within the limits of possible error, it should not be given too much importance. Furthermore, it offsets the slight difference in mortality that favored the sweet milk feeding (table IX).

The results of the entire investigation of milk feeding, which extended over a period of almost three years, fully justify the conclusion that milk is an important factor of diet in as far as mortality and growth are concerned. Whether milk is fed as sweet, ordinary sour, or *Bacillus bulgaricus* milk, the effect is essentially the same. Hence, milk as such possesses one or more ingredients which are of unique significance. No experiments have been undertaken, in connection

<sup>8</sup> Rettger, Kirkpatrick, and Card, *loc. cit.*



with the general problem of milk feeding, to determine what these physiologically active substances are. Some reference to the work of Osborne and Mendel will be of special interest here.

These investigators have shown that butter fat, when fed to rats which failed to complete their growth on a diet consisting of pure proteins, starch, protein-free milk, and commercial lard, enabled the rats to regain their lost growth.<sup>9</sup> They have also acquired an abundance of evidence to indicate that lactalbumin<sup>10</sup> is of particular value in fostering growth. In other words, lactalbumin furnished the necessary factors in their experiments which are required for normal growth. Since lactalbumin is rich in both tryptophane and lysine, it is but natural to assume that this milk protein owes its unique dietary properties to these two substances that it contains in its molecule. Osborne and Mendel have apparently demonstrated that tryptophane is necessary for maintenance, but wholly inadequate to produce growth, and that lysine is indispensable for growth.

## II. THE INFLUENCE OF MILK FEEDING ON THE CHARACTER OF THE INTESTINAL FLORA.

That the value of the feeding of milk does not lie in acids that may be present in the milk, nor in acid-producing bacteria which as a rule constitute a large part of the organisms occurring in milk, has been clearly demonstrated in the foregoing investigations. These results are not in harmony with the ideas of many, who hold that sour milk, or the acid product which is prepared with the aid of the so called "Bulgara" or "Bulgaricus tablets," is of great dietary importance because of the presence of these acid-producing bacteria, or of their acid products.

After experimenting upon himself, Leva<sup>11</sup> arrived at the conclusion that the ingestion of Metchnikoff's lactobacilline brought about an acclimatization of *B. bulgaricus* in the intestine. This organism was found to be present in the feces on and after the fifth day following the use of the tablets. He also observed that during the investigation the amounts of aromatic oxyacids, hippuric acid, and phenol in the urine were decreased.

In an extensive investigation in which thirty different hospital patients were employed as subjects, besides the author himself, Cohendy<sup>12</sup> was led to conclude that the use of milk which is soured by *B. bulgaricus* causes a marked transformation of the intestinal flora, which is made apparent by the deodorization

<sup>9</sup> Osborne, Mendel, Ferry, and Wakeman, *loc. cit.*, 1913, xv, 311; 1913-14, xvi, 423; 1914, xvii, 401.

<sup>10</sup> Osborne, Mendel, Ferry, and Wakeman, *loc. cit.*, 1914, xvii, 325.

<sup>11</sup> Leva, J., *Berl. klin. Wchschr.*, 1908, xlv, 922.

<sup>12</sup> Cohendy, M., *Compt. rend. Soc. de biol.*, 1906, lviii, 602.

of the feces and by a decrease in the amounts of conjugate sulphates in the urine. He ascribed the change to the lactic acid bacillus, without due regard to the milk itself.

Belonovsky<sup>13</sup> employed white mice. The diet consisted of sterilized wheat grain to which cultures of *B. bulgaricus* had been added. Twelve days after the beginning of the experiment a marked change was noted in the character of the intestinal flora. There was a large increase in the number of Gram-positive organisms, and a corresponding decrease in gas-producers. Similar results, although less pronounced, were obtained with mice that were fed sterilized wheat and killed cultures of *B. bulgaricus*. The author further states that in young nursing mice the intestinal flora presented practically the same microscopic appearance as in the older mice which received the wheat and *bulgaricus* cultures. Direct microscopic examination of the feces of the mice that received the *bulgaricus* bacillus cultures failed to reveal a predominance of the *bulgaricus* type over other organisms. In fact, it is to be inferred that bacilli of this description were very few. The writer believes that the action of *B. bulgaricus* is not due entirely to the bacilli, or to the lactic acid, but to other products as well.

The opinions cited above are essentially the same as those which Metchnikoff<sup>14</sup> announced and reiterated from time to time, and which have apparently gained a firm hold in the minds of many scientists as well as laymen. It is claimed that the wonderful accomplishments of *B. bulgaricus* are closely linked with its power to destroy or eliminate harmful bacteria from the intestinal tract, particularly those having putrefactive properties. Hence, by its ability to prevent autointoxication, it serves as an important therapeutic agent, even to the extent of preventing premature old age.

In the investigations upon which this part of the paper is based at least seventy-five white rats were employed. A moderate number of experiments was conducted also on chicks of different ages and on adult fowls. The work was the natural outcome of the earlier investigation of Rettger and Horton<sup>15</sup> on the intestinal flora of white rats kept on experimental and ordinary mixed diets.

Special emphasis has been given to organisms which belong to the type of *Bacillus bifidus* (Tissier) and of *Bacillus acidophilus* (Moro), though other types of intestinal organisms were not overlooked or neglected when they were present in appreciable numbers. *Bacillus acidophilus* assumed much prominence in the earlier feeding experiments which were carried on in connection with the elaborate investigations of Osborne and Mendel on the influence of pure pro-

<sup>13</sup> Belonovsky, J., *Ann. de l'Inst. Pasteur*, 1907, xxi, 991.

<sup>14</sup> Metchnikoff, E., *Prolongation of Life*, translated by Mitchell, P. C., London, 1907.

<sup>15</sup> Rettger, L. F., and Horton, G. D., *Centralbl. f. Bakteriol., 1te Abt., Orig.*, 1914, lxxiii, 362.

tein diets on growth. When the rats were transferred from the ordinary mixed to the pure protein diets a marked transformation in the character of the intestinal flora invariably took place. The flora became more simplified, very few types of bacteria being found after an interval of two or three days following the change of food. Organisms belonging to the *acidophilus* group always became numerous, and at times were so prominent as to exclude all other types except *Bacillus bifidus*. This change in the intestinal flora was undoubtedly independent of the pure proteins, but, as later experiments have indicated, bore a definite relation to the protein-free milk which formed a part of the pure protein diet.

The experiments<sup>16</sup> recorded here will be presented under three different heads; namely, the influence of milk feeding, the influence of carbohydrate feeding, and the influence of the ingestion of *Bacillus bulgaricus*, on the intestinal flora. The results of only a limited number of experiments which are typical will be given in detail.

The methods employed were essentially the same in the different series of experiments. The rats were kept in cages which were constructed of rather coarse wire net and supported at some distance over a tray which was covered daily with clean paper, so that the feces could be collected with as little chance of contamination as possible. Samples of feces were collected at regular intervals, sometimes daily. From 0.2 to 0.4 of a gram of the samples was vigorously shaken in test-tubes containing eight to ten cubic centimeters of sterile water and a little broken glass until a uniform suspension was obtained. From these suspensions agar plates and Veillon tubes were prepared in the desired dilutions. Smears were made also on slides and stained by the Gram method.

For plate-pouring different kinds of agar medium were used; namely, plain agar, dextrose agar (1 per cent. dextrose), acid dextrose agar (1 per cent. dextrose, 0.5 per cent. acetic acid), and neutral whey agar (neutral whey 1 liter, peptone 10 grams, agar 15 grams, and Liebig's meat extract 3 grams). The dextrose agar was used at practically all times, especially in the Veillon tubes.

<sup>16</sup> These experiments were conducted largely by Thomas G. Hull, and appear in part in another paper (Hull, T. G., and Rettger, L. F., *Centralbl. f. Bakteriologie Abt., Orig.*, 1914, lxxv, 219).

All the rats were fed bread and green vegetables as the basic diet. In the different experiments the special agents to be studied were added to the basic diet. In several instances the bread was ground in a meat grinder. Numerous examinations of the feces of white rats that had been subsisting upon bread and green vegetable food for at least three or four days had shown that the intestinal flora were of the usual mixed type, the absence, or but a small number, of *Bacillus acidophilus* and *Bacillus bifidus* being particularly noticeable. Hence, such a diet was of much value in bringing the flora to a standard or uniform basis as a definite starting or end point in the different experiments.

#### MILK FEEDING.

In none of the experiments was the milk supplied until examination of the feces demonstrated that *Bacillus acidophilus* and *Bacillus bifidus* were absent from the feces, or that they were comparatively rare. Furthermore, control rats which received only the bread and vegetable diet were employed along with the others. The milk, which was usually whole milk, was poured over the dried bread crumbs or in the water dish in place of the water. The following experiments are given here as examples.

*Experiment A.*—Rats 1, 2, and 3 received 20 to 30 c.c. of milk three times a week for four weeks, in addition to the basic diet, while rats 4, 5, and 6 were given the bread and vegetable food only. During the entire period *B. acidophilus* was practically absent from the feces of the control rats, whereas in the milk-fed rats it was abundant after the first few days and continued so until the milk feeding was discontinued, after which the *acidophilus* group again disappeared.

*Experiment B.*—Rats 2 and 3 received, in addition to the bread and vegetable diet, 10 c.c. of milk daily for ten days. After the first three days *B. acidophilus* appeared, and numerous colonies of this organism were obtained on the agar plates. It was also very abundant in smears prepared from the feces, although various other types were present in small numbers.

*Experiment C.*—Rat 23 was supplied with 50 c.c. of milk daily for forty days. Within two to three days *B. acidophilus* became very numerous, which was shown by the large numbers of colonies on the agar plates, and by the small number of other types, excepting *B. bifidus*, in the microscopic mounts. Besides *B. acidophilus*, the Gram stained smears contained numerous rods which were smaller than the others, and which showed evidence of branching (*B. bifidus*). In the Veillon tubes *B. acidophilus* was quite abundant throughout the length of the tubes in the form of the typical shaggy colonies, while the smooth, disc-like colonies of *B. bifidus* appeared in sharp contrast in the deeper portions of the

tubes. As the experiment progressed *B. bifidus* increased in numbers, and in a large measure supplanted *B. acidophilus*.

At the termination of the forty days' period but little milk was taken by the rat, and although milk was supplied for the next ten days very little was consumed. As a consequence, *B. bifidus* disappeared from the intestine, and the number of *B. acidophilus* was greatly reduced, while other types again became more common.

Postmortem examinations were made of rats 2 and 3 after they had been on the bread, vegetable, and milk diet for ten days. Cultural and microscopic tests of the gastro-intestinal tract revealed the presence of *B. acidophilus* in large numbers, and in almost pure form, in the small intestine. It was also found to be abundant in the large intestine, but here it was mixed with various other organisms.

Numerous other experiments on milk feeding, both with white rats and with chicks of different ages, have given similar results. Sufficient evidence is at hand, therefore, to show that profound alterations in the character of the intestinal flora, at least of the white rat and the common domestic fowl, are brought about by the addition of milk to a diet which has established a flora in which the *acidophilus* and *bifidus* types of bacteria are few or absent. In this transformation the usual mixed flora are, in a large measure at least, supplanted by ones rich in *Bacillus acidophilus* or *Bacillus bifidus*, or both of these organisms. A limited number of experiments on man have given results similar to the above. The complete transformation of types is, however, not accomplished so readily as in the rat or in fowls.

Since *Bacillus acidophilus* and *Bacillus bifidus* are both carbohydrate-consuming organisms, and have but slight proteolytic properties, it seemed but natural to look for a possible explanation of the above results in the lactose content of the milk. For this purpose numerous feeding experiments were conducted on white rats with seven different carbohydrates.

#### CARBOHYDRATE FEEDING.

The following carbohydrates were used: lactose, sucrose, maltose, dextrose, levulose, galactose, and dextrine. At first each carbohydrate was fed in the form of a concentrated aqueous solution, which was poured over the bread crumbs; later three to four grams of the carbohydrate in question were placed on the bread and moistened

with a small amount of water. The lactose feeding experiments were carried on for periods varying from one to seven weeks.

*Experiment A.*—Rat 13 was fed lactose for fifteen days. On the third day *B. acidophilus* was observed in appreciable numbers in the smears and agar plates prepared from the feces, after which it practically disappeared, and *B. bifidus* was found to be present in almost pure form in the smears.

*Experiment B.*—Rat 12 received lactose for thirty days, but little change was observed in the intestinal flora, neither *B. acidophilus* nor *B. bifidus* being present in appreciable numbers in the slides, plates, or Veillon tubes. No explanation can be given for this irregularity in the results, except that the rat was peculiarly resistant to bacterial changes in the intestine, which as a rule are readily induced by change of diet.

*Experiment C.*—Rats 16 and 17 obtained milk sugar for eleven days. *B. bifidus* became quite prominent on the second day and continued to the end of the period. *B. acidophilus* also was present in small numbers.

*Experiment D.*—Rat 35 was given lactose for thirteen days. *B. acidophilus* became very abundant at the outset, but soon diminished in numbers, while *B. bifidus* increased to such an extent as to exclude almost all other forms.

*Experiment E.*—Rat 26 received lactose for fifty days. For a short time the intestinal flora consisted largely of *B. bifidus* and, to a lesser extent, of *B. acidophilus*. Later *B. bifidus* was practically the only organism present.

*Experiment F.*—Rats 21 and 22 were fed milk sugar for sixteen days; Nos. 14, 15, and 16 for twenty days; Nos. 17 and 19 for eight days; Nos. 23, 27, and 28 for seven days, and No. 9 for ten days. In every instance *B. bifidus* became very abundant soon after the addition of the lactose to the diet. In general, the following changes took place in the intestine. By the second or third day *B. acidophilus* and *B. bifidus* began to make their appearance. After having attained a certain maximum *B. acidophilus* gradually decreased in number, and was practically absent at the close of the period, with very few exceptions. *B. bifidus* increased very fast, however, and in every case it became the predominant form.

Several lactose rats were killed for the purpose of making bacteriological examinations of the digestive tract. In rat 9 no colonies of *B. acidophilus* or *B. bifidus* were obtained from the stomach. A small number of the *acidophilus* type was found in the duodenum, while throughout the remainder of the intestine *B. bifidus* was quite abundant. In rat 26 (fed lactose for fifty days) *B. bifidus* was present in large numbers in the stomach, the small intestine below the duodenum, and in the large intestine. *B. acidophilus* was found also, but to a limited extent. Rat 15, which required an unusual length of time for *B. bifidus* to establish itself, contained *B. acidophilus* in the stomach and throughout the small and large intestine, while *B. bifidus* was plentiful in the ilium and in the large intestine.

The foregoing experiments demonstrate that both milk and lactose, when fed in sufficient quantities, have a pronounced influence on

the character of the intestinal flora, each bringing about a profound change in which the ordinary mixed flora give way to ones that are more simplified and which are made up largely of *Bacillus acidophilus* or *Bacillus bifidus*, or both. That milk owes this property to the lactose which it contains can hardly be doubted. Since almost half of the total solids of milk is milk sugar, namely, about 5 per cent., it is not surprising that milk when fed even in small amounts has such a marked influence on the flora of the intestine.

A large number of feeding experiments was conducted in which sucrose, maltose, dextrose, levulose, galactose, and dextrine were used in their turn. Very little or no change could be observed in the intestinal flora of any of the rats. In order to determine whether the negative results were due to an inhibiting influence of the different carbohydrates, a limited number of feeding experiments was carried out in the following manner. Rats which had been receiving lactose, and which exhibited the characteristic flora of milk- or lactose-fed rats, were given, besides the usual amount of milk sugar (three to four grams daily), an equal amount of the carbohydrate in question. No decrease could be observed in the numbers of organisms of the *acidophilus* and *bifidus* types, nor was there any other apparent change. The failure, therefore, of the intestinal bacteria to be affected by any of these carbohydrates, except lactose, can not be ascribed to any inhibitory action which they or their products may exert.

Thus far one significant fact has been demonstrated repeatedly; namely, that diet may be a very important factor in determining the character of the intestinal flora.

The above results are in accord with those of Sittler,<sup>17</sup> who found that when lactose is fed to children that subsist on cow's milk the typical *bifidus* flora of breast-fed infants are present. Similar results were obtained with so called "malt soup." On the other hand, cane sugar did not react in this way. The limited number of experiments which was carried on with dextrose seemed to indicate that it played the same part as lactose. This claim could not be substantiated by us.

Quite recently Distaso and Schiller<sup>18</sup> claim to have shown that the feeding of milk, lactose, or dextrine to white rats which have been on a diet of bread and meat brings about a change in the intestinal flora from the usual mixed to

<sup>17</sup> Sittler, P., *Centralbl. f. Bakteriol., 1te Abt., Orig.*, 1908, xlvii, 14.

<sup>18</sup> Distaso, A., and Schiller, J., *Compt. rend. Soc. de biol.*, 1914, lxxvi, 243.

the *bifidus* type. No mention in their brief publication is made of *B. acidophilus*. Our results on the feeding of dextrine are not in harmony with those just cited. The difference may be due to the dextrine employed. Further work to determine the influence of dextrine, and starches as well, is under way.

It is surprising that so little attention has been given in past investigations to the occurrence of *B. acidophilus* (Moro),<sup>19</sup> and to the conditions that influence its numbers in the intestine. In our work on the intestinal flora of the albino rat and of the common domestic fowl, organisms of the *acidophilus* type have been met with almost constantly. In the feces of man and of guinea pigs we have found them frequently also, and at times in considerable numbers. While *B. bifidus* (Tissier)<sup>20</sup> was in most instances associated with *B. acidophilus*, the latter was, as a rule, much more prominent. Exceptions to this rule occurred in some of the lactose feeding experiments.

#### THE INFLUENCE OF THE INGESTION OF BACILLUS BULGARICUS ON THE CHARACTER OF THE INTESTINAL FLORA.

The feeding of *Bacillus bulgaricus* was accomplished in two ways. In some instances the rats received milk cultures of the organism. These cultures were prepared by inoculating sterilized skimmed milk with the organism and incubating for twenty-four hours at 37° C. By the other method large surfaces of dextrose agar were inoculated with *Bacillus bulgaricus*. After an incubation period of twenty-four hours the surface growth was washed off with sterile water. The water suspensions were fed as such in small dishes, or they were poured over the bread crumbs. In all the *bulgaricus* feeding the regular basic diet of bread and vegetables was employed. In some of the experiments sterile milk was fed to one or two of the rats, instead of the inoculated milk or the bacterial suspensions, also the washings of sterile agar.

It was difficult at the outset to distinguish between *Bacillus bulgaricus* and *Bacillus acidophilus*, and especially the colonies on dextrose agar. Certain marks of distinction were noted, however, but the identification was never regarded as satisfactory without the milk acidification test. Since *Bacillus bulgaricus* rapidly produces enough acid to coagulate the milk, and a recently isolated *acidophilus* strain does so only after more prolonged incubation, this test was of considerable importance.

Special precautions had to be taken to minimize the chances of

<sup>19</sup> Moro, E., *Jahrb. f. Kinderheilk.*, 1900, lii, 38.

<sup>20</sup> Tissier, H., *Ann. de l'Inst. Pasteur*, 1905, xix, 109; 1908, xxii, 189.



contamination of the feces by *bulgaricus* bacilli that were supplied with the food. This was accomplished with no little effort.

*Experiment A.*—Six rats were employed. Rats 1 and 4 received the basic diet of bread and vegetables together with a watery suspension of *B. bulgaricus*; rats 2 and 5 were given the washings of sterile agar, in addition to the regular diet, while Nos. 3 and 6 received the bread and vegetable only. The experiment continued for four weeks, with daily feeding. *B. bulgaricus* was observed but twice in the feces of the rats that were abundantly supplied with it. Furthermore, no differences could be detected between the flora of rats 1 and 4 and those of the four rats which were not supplied with the organism in question. In all the rats the typical mixed flora prevailed.

In postmortem examinations conducted on rats 1 and 4 eighteen hours after the last feeding of *B. bulgaricus*, an organism resembling the *bulgaricus* bacillus was found in very small numbers in the stomach and duodenum, while from the remainder of the intestine not a single colony of this organism could be obtained.

*Experiment B.*—Rat 21 received the stock diet together with a washed culture (water suspension) of *B. bulgaricus*; No. 22 was given the regular diet plus 50 c.c. of milk that was fermented by *B. bulgaricus*, and rat 23 the usual diet plus 50 c.c. of sterilized milk. From the feces of rat 21 single colonies resembling those of *B. bulgaricus* were obtained three times; from No. 22 individual colonies of the *bulgaricus* type were obtained but four times, while in the plates from rat 23 no colonies of *B. bulgaricus* were detected, though colonies of *B. acidophilus* were quite abundant. This experiment continued over a period of four weeks.

*Experiment C.*—Rat 13 received bread and lettuce plus the water suspension of *B. bulgaricus*; No. 32 was given the same diet plus 50 c.c. of sterile milk and the water suspension; and rat 23 the same basic diet plus 50 c.c. of sterile milk. *B. bulgaricus* could at no time be found in the feces of the rats (13 and 32) that were fed the living bacilli; nor was there any apparent difference between the intestinal flora of rats 32 and 23. This experiment lasted ten days.

On postmortem examination one of the three rats that were given *B. bulgaricus* in water suspension failed to show the presence of this organism in any portion of the digestive tract; in the second a very small number was recovered from the jejunum and ilium only. The third rat had been receiving lactose for some time. *B. bulgaricus* could not be recovered from it, while *B. bifidus* was found in large numbers.

In all the rats that received milk, whether it was sterile or inoculated with *B. bulgaricus*, *B. acidophilus* and *B. bifidus* were at all times more or less prominent, as in the previous milk-feeding experiments. On the other hand, in spite of the large numbers of *bulgaricus* bacilli that were fed to some of the rats, very few could be recovered from the feces, and at no time did this organism establish itself in the intestine, even after the daily ingestion of the bacilli for periods of from ten days to four weeks.

These observations are in accord with those of Luerksen and Kühn,<sup>21</sup> who failed to establish *B. bulgaricus* in the intestine of man by the continued use of

<sup>21</sup> Luerksen, A., and Kühn, M., *Centralbl. f. Bakteriol., 2te Abt.*, 1908, xx, 234.

yoghurt, and with the views of Distaso and Schiller,<sup>22</sup> who claim to have shown quite recently that the ingestion of large numbers of this organism by rats which are receiving milk or lactose does not result in its acclimatization in the intestine, and that *B. bifidus* is the predominating type of bacteria.

Oehler<sup>23</sup> conducted some feeding experiments with yoghurt on mice and monkeys, and stated that the *bulgaricus* bacillus could be demonstrated with ease in the feces during the yoghurt feeding period. Within two to three days after the yoghurt feeding was discontinued, *B. bulgaricus* again disappeared from the intestine. It should be borne in mind that large quantities of milk as such were supplied in the form of the yoghurt, and hence the conditions for the development of *B. acidophilus* were most favorable. Some doubt may be felt, therefore, as to whether Oehler's *B. bulgaricus* may not have been the Moro bacillus. It is conceivable, however, that any species of organism may be recovered in limited numbers from the feces if ingested in extremely large numbers.

Herter and Kendall<sup>24</sup> found that in a monkey which was killed three to four hours after it had been fed 500 c.c. of milk that had been well soured by bacillac very few *bulgaricus* bacilli were present in the large intestine. In an earlier experiment on this monkey they had failed to detect *B. bulgaricus* in the feces after feeding the sour milk (bacillac) daily for three days. These same authors state that the intestinal flora of cats and monkeys were rapidly altered when a diet of meat or eggs was followed by one of milk and dextrose. The most important change was the substitution of an acidophilic, non-proteolyzing type of flora for one which was strongly proteolytic.

Even *B. coli*, especially a foreign strain, appears to be unable to establish itself in the intestine as the result of the feeding of this organism. This was clearly demonstrated by Rettger and Horton<sup>25</sup> who supplied white rats which were on diets containing pure protein as the only available nitrogenous food with vast numbers of colon bacilli. In one of the experiments a very small increase of *B. coli* took place in the intestine; while in another there was no perceptible increase, although *B. coli* was fed at frequent intervals for a period of almost four weeks.

Similar results were obtained by Seiffert<sup>26</sup> who showed that a strain of *B. coli* isolated from another species failed to inure itself to the conditions which obtain in the human intestine after being taken into the system *per os*. A strain which had been isolated from the same person, however, rapidly multiplied and was found in abundance in the intestine. Raubitschek<sup>27</sup> also claims to have demonstrated that foreign organisms are unable to establish themselves in the intestine, except after at least partial immunization of the host to the particular organisms.

Kulka<sup>28</sup> conducted a series of experiments on man and rabbits. His results appear to be very decisive, demonstrating that *B. metchnikovi* and *B. prodigiosus*,

<sup>22</sup> Distaso and Schiller, *loc. cit.*

<sup>23</sup> Oehler, R., *Centralbl. f. Bakteriol., 2te Abt.*, 1911, xxx, 149.

<sup>24</sup> Herter, C. A., and Kendall, A. I., *Jour. Biol. Chem.*, 1908-9, v, 293.

<sup>25</sup> Rettger and Horton, *loc. cit.*

<sup>26</sup> Seiffert, G., *Deutsch. med. Wchnschr.*, 1911, xxxvii, 1064.

<sup>27</sup> Raubitschek, H., *Virchows Arch. f. path. Anat.*, 1912, ccix, 209.

<sup>28</sup> Kulka, W., *Arch. f. Hyg.*, 1914, lxxxii, 337.

when introduced either by mouth or by subcutaneous, intravenous, or intraperitoneal injection, do not appear in the feces. Mitchell and Bloomer<sup>29</sup> obtained similar results in the common domestic fowl with the typhoid bacillus.

#### GENERAL CONCLUSIONS.

Throughout the investigations upon which a large part of this paper is based the favorable influence of milk feeding on mortality and growth was most apparent. Mortality from all causes was frequently reduced to at least one-half of what obtained among the chicks that received no milk, while the milk-fed chicks in some experiments gained twice as much in weight as those that were without this article of diet. Although the influence of milk feeding was less pronounced on the mortality of chicks that were artificially infected with *Bacterium pullorum*, quite an appreciable difference in mortality was always noted if the milk was fed at least one or two days before the first administration of the bouillon cultures of the organism in question.

Practically the same results were obtained, whether sweet or sour milk was fed, and no differences could be observed in the relative value of ordinary sour milk and of the so called *bulgaricus* product. Hence, the unique properties of this food exist in the milk as such, rather than in any milk acids or milk bacteria that may be present.

Milk and lactose diet exert a very important influence on the character of the intestinal bacteria, especially in white rats and in the common domestic fowl. Within a few days after the ingestion of milk or lactose a transformation of the flora takes place in which the usual mixed bacterial flora give way to ones that are more simplified, and in which *Bacillus acidophilus* and *Bacillus bifidus* are, as a rule, prominent. It is to be assumed that milk has this influence in virtue of the large amount of lactose which it contains. Other carbohydrates, besides milk sugar, failed to bring about such a transformation.

The ingestion of foreign bacteria, even in large numbers, does not of itself bring about an elimination or displacement of the common intestinal microorganisms. Vastly more important is the influence of diet, especially milk and lactose. The feeding of Bulgara

<sup>29</sup> Mitchell, O. W. H., and Bloomer, G., *Jour. Med. Research*, 1914, xxxi, 247.

tablets or other preparations which contain as the supposedly active agent the bacillus of Metchnikoff and Mazé, without due regard to the use of milk, can, therefore, be of little, if indeed of any, value. The beneficial effects which it is claimed have been derived from the use of yoghurt, and other oriental sour milk products have in all probability been due to the milk as such, rather than to the bacteria which they contained. This view is strongly supported by the extensive milk feeding experiments on chicks which are recorded in this paper, and also by the results which show the influence of milk and of lactose feeding on the intestinal flora of white rats and of the common domestic fowl.

## CONCENTRATION OF THE PROTECTIVE BODIES IN ANTIPNEUMOCOCCUS SERUM BY MEANS OF SPECIFIC PRECIPITATION.\*

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Previous work has shown that the occurrence of a specific precipitin reaction between a serum of one animal species and its antiserum may result in a very definite action on normal or immune antibodies contained in the first (precipitinogenic) serum.

Such studies were inspired by the work of Camus and Gley (1), Kossel (2), and Bordet (3), who had shown that antihemolysins were formed by immunization against a hemolytic serum, and were designed to determine whether similar antibodies could be produced to antitoxins, agglutinins, precipitins, and the like.

Kraus and Eisenberg (4) succeeded in producing an antilactoserum, the action of which was apparently due to the carrying down of the precipitin of the lactoserum with its precipitinogen which had been allowed to interact with the antilactoserum. In other words, the serum of a rabbit immunized against milk when precipitated by the serum of a goat immunized against rabbit serum removes the precipitin for milk from the lactoserum. The first combination was for some reason not effective when a dog-antirabbit serum was employed. These same authors failed, however, to demonstrate fixation of tetanus antitoxin or of typhoid agglutinin in horse serum after precipitating the horse serum by means of rabbit-antihorse serum. Their failure to remove the antitoxin was, however, due to the excess of precipitinogen (horse antitoxin) employed, an inhibiting factor to precipitate formation that was then unknown. This difficulty was overcome by Dehne and Hamburger (5), who found that diluted (1:500) horse antitoxin was entirely removed from the supernatant fluid on producing a precipitate with rabbit-antihorse serum. These experiments were fully corroborated by Kraus and Pribram (6), Hamburger (7), and von Eisler and Tsuru (8), and analogous facts were produced in respect to the fixation of diphtheria antitoxin in horse serum by Weill-Hallé and Lemaire (9) and by Atkinson and Banzhaf (10). Experiments which indicate that a similar removal of antitoxin takes place in the body of immunized animals were performed with diphtheria antitoxin by Sacharoff (11) and with tetanus antitoxin by Dehne and Hamburger (5).

Some difference of opinion exists as to the carrying down of agglutinins with a precipitinogen when antiserum is added, the results obtained doubtless depending on technical differences. Thus, Kraus and Eisenberg (4) at first failed to remove immune agglutinins, but later Kraus and Pribram (6) succeeded. Von Eisler and Tsuru (8) and Landsteiner and Prašek (12) brought down normal

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agglutinins, but the latter at times failed with immune agglutinins. Wassermann and Bruck (13) failed to remove antityphoid protective bodies by precipitation with rabbit-antigoat serum, whereas some such removal is indicated by the experiments of Walker (14) performed *in vivo*. Zebrowski (15) failed to remove the hemolytic sensitizer (amboceptor) by similar treatment.

These facts are of significance in considering the mode of action of antisensitizers (antiamboceptors) as bearing on the relations of lysins to "Bordet's fixation bodies," and particularly as bearing on the relation of the fixation reaction to the formation of specific precipitates (16). We do not propose, however, to discuss any of these interesting and somewhat puzzling questions at this time, but to direct attention to the one fact that an antiserum (antitoxin, agglutinating serum) when employed as a precipitinogen by the addition of an homologous antiserum (precipitin) may yield to the precipitate its entire content of antibody.

It is the reverse of this reaction which we wish to describe, employing antipneumococcus serum as precipitin and producing a precipitate by the addition of an extract of the pneumococcus. We find that such a precipitate brings down more or less completely the antibodies which protect animals against pneumococcus infection. Owing to a corresponding reversal of the relative dosage of the reacting substances, this reaction becomes at once of practical as well as of theoretical interest. It enables us not only to concentrate the specific antibodies of the antipneumococcus serum in small volume, but, as we shall see, in a fluid of extremely low protein content.

Although bacterial precipitins were the first to be described (Kraus, 1897 (17)), interest in them has for practical reasons been far exceeded by that taken in the serum and protein precipitins later described by Tchistovitch (18) and by Bordet (19). Neufeld (20) first described a precipitin for pneumococcus extract in the serum of specifically immunized animals. His observations were extended by Wadsworth (21), by Panichi (22), by Heyrovský (23), and by Norris (24).

In our own work we have employed potent antipneumococcus sera produced in horses by prolonged treatment with dead and living cultures of either type I or type II (25) strains of pneumococcus. Such sera agglutinate specifically the type of pneumococcus employed for

immunization in low dilutions, but have no effect on other types. They further protect mice in a dose of 0.2 of a cubic centimeter against 0.1, or rarely 0.2, of a cubic centimeter of a twenty-four hour bouillon passage culture of pneumococcus of which 0.000,001 of a cubic centimeter suffices to kill controls.

As antigen (precipitinogen) we have used water-clear extracts of dried and ground pneumococci obtained by precipitating considerable amounts of washed concentrated bouillon cultures by the addition of equal parts of 95 per cent. alcohol, or, in later experiments, by adding them to ten volumes of acetone (26) (Kahlbaum's C. P.).<sup>1</sup> These extracts were made in the manner that Gay and Claypole (27) have described for producing plain typhoid vaccines, and are made by suspending weighed amounts of ground culture in carbolated (0.5 per cent.) saline solution in the proportion of one milligram to one cubic centimeter, shaking at intervals at a temperature of 37° C. for from three to six hours, and then allowing them to remain in the ice box over night. On the following day the suspended mixture is vigorously centrifugalized until the supernatant fluid is cloudless and water-clear, or at most slightly opalescent. Such supernatant fluids from ground pneumococci are very toxic for mice, distinctly more so than the bacterial sediment from which they have been separated.

This clear antigen gives a distinct precipitin reaction with one cubic centimeter of strong immune serum in a dose of 0.01 of a cubic centimeter or less, when read from the sediment after twenty-four hours at room temperature. The maximal precipitate and rapidity of reaction are produced by the addition of from 0.3 to one cubic centimeter of the antigen when added in a single dose. We have as yet no information at hand as to the zone of inhibition or resolution of the precipitate in an excess of antigen. In producing precipitates to test for protective purposes our method has been to endeavor to produce the maximum precipitate from a given amount of immune serum. For this purpose we have added the antigen in divided doses, allowing the cloudy or flocculent precipitate to form for from a few minutes to several hours, centrifugalizing, and then

<sup>1</sup> This was done at the suggestion of Dr. Van Slyke.

adding more precipitinogen to the supernatant serum until no further cloud is produced.

We have by no means fully determined the ideal conditions for producing the maximal precipitate, or perhaps, more correctly speaking, the optimal protein complex in a precipitate, as is indicated by the considerable variations in the total nitrogen determinations of our washed precipitates. Nor have we in all instances succeeded in bringing down all the protective antibodies in our precipitates. The fact, however, that we have repeatedly found such precipitates, washed free of serum, to contain as much protective value in aliquot parts as the immune serum, seems to us to justify this communication, pending a greater uniformity of results.

#### EXPERIMENTAL.

*Experiment 1.*—To 20 c.c. of antipneumococcus serum (type I, lot 14, Dec. 6, 1913) were added 6 c.c. of clear carbolated extract of dried pneumococci. An immediate voluminous precipitate occurred. This was centrifugalized five minutes later and another 6 c.c. of antigen were added to the supernatant fluid. A slow cloudiness appeared which was allowed to form for two hours at 37° C. The second supernatant fluid in a dose of 1 c.c. gave no further precipitate with doses of from 0.1 to 1 c.c. of antigen. The combined precipitates were shaken in 20 c.c. of carbolated saline, centrifugalized, and resuspended. 20 c.c. of the same specimen of antipneumococcus serum were diluted *pari passu* with the above by addition of two doses of 6 c.c. each of carbolated saline solution instead of antigen.

One-half the above washed precipitate was dissolved by 2 c.c. of  $\frac{N}{10}$  sodium hydrate. It was further diluted with saline until 0.5 c.c. of the solution corresponded to the precipitate derived from 0.2 c.c. of original serum. The undissolved precipitate, the original diluted serum, and the exhausted (precipitin-free) serum were also made up to corresponding volume (0.5 c.c. equals 0.2 c.c. of original serum).

A Kjeldahl determination by Mr. Cullen gave a total nitrogen in the undissolved precipitate, indicating a protein content of 0.34 per cent.

With these mixtures the following experiments were performed on mice (tables I, II, and III).



TABLE I.

*A. Protective Experiment with Serum and Derivatives Mixed in a Fixed Amount with Decreasing Amounts of Dilutions of a Twenty-Four Hour Bouillon Culture of Pneumococcus, Type I, 96-1,<sup>2</sup> in a Total Volume of 0.5 C.C. The Mixtures Were Injected Intra-peritoneally in Mice.*

Mouse.	Protective fluid.	Culture.		Result.
1	Control	0.0001	c.c.	Died, 45 hrs.
2	Control	0.00001	c.c.	Died, 45 hrs.
3	Control	0.000001	c.c.	Died, 45 hrs.
4	Original serum 0.2 in 0.5 c.c.	0.5	c.c.	Died, 45 hrs.
5	Original serum 0.2 in 0.5 c.c.	0.1	c.c.	Survived.
6	Original serum 0.2 in 0.5 c.c.	0.01	c.c.	Died, 34 hrs.
7	Original serum 0.2 in 0.5 c.c.	0.001	c.c.	Survived.
8	Exhausted serum 0.2 in 0.5 c.c.	0.5	c.c.	Died, 20 hrs.
9	Exhausted serum 0.2 in 0.5 c.c.	0.1	c.c.	Died, 45 hrs.
10	Exhausted serum 0.2 in 0.5 c.c.	0.01	c.c.	Survived.
11	Exhausted serum 0.2 in 0.5 c.c.	0.001	c.c.	Survived.
12	Whole washed precipitate 0.2 in 0.5 c.c.	0.5	c.c.	Died, 45 hrs.
13	Whole washed precipitate 0.2 in 0.5 c.c.	0.1	c.c.	Survived.
14	Whole washed precipitate 0.2 in 0.5 c.c.	0.01	c.c.	Survived.
15	Whole washed precipitate 0.2 in 0.5 c.c.	0.001	c.c.	Died, 45 hrs.
16	Dissolved precipitate 0.2 in 0.5 c.c.	0.5	c.c.	Died, 45 hrs.
17	Dissolved precipitate 0.2 in 0.5 c.c.	0.1	c.c.	Survived.
18	Dissolved precipitate 0.2 in 0.5 c.c.	0.01	c.c.	Survived.
19	Dissolved precipitate 0.2 in 0.5 c.c.	0.001	c.c.	Died, 4 dys.

TABLE II.

*B. Protective Experiment with Fixed Dose of Culture and Decreasing Doses of Serum and Serum Derivatives.*

Mouse.	Protective fluid.	Total volume 0.5 c.c.	Culture, Pneumococcus, type I, 98-1.		Result.
1	Original serum 0.1	c.c.	0.05	c.c.	Survived.
2	Original serum 0.05	c.c.	0.05	c.c.	Survived.
3	Original serum 0.025	c.c.	0.05	c.c.	Survived.
4	Original serum 0.0125	c.c.	0.05	c.c.	Survived.
5	Original serum 0.00625	c.c.	0.05	c.c.	Died, 14 dys.
6	Dissolved precipitate 0.1	c.c.	0.05	c.c.	Survived.
7	Dissolved precipitate 0.05	c.c.	0.05	c.c.	Survived.
8	Dissolved precipitate 0.025	c.c.	0.05	c.c.	Survived.
9	Dissolved precipitate 0.0125	c.c.	0.05	c.c.	Survived.
10	Dissolved precipitate 0.00625	c.c.	0.05	c.c.	Survived.
11	Exhausted serum 0.1	c.c.	0.05	c.c.	Survived.
12	Exhausted serum 0.05	c.c.	0.05	c.c.	Died, 36 hrs.
13	Exhausted serum 0.025	c.c.	0.05	c.c.	Died, 36 hrs.
14	Control		0.05	c.c.	Died, 20 hrs.
15	Control		0.0001	c.c.	Died, 18 hrs.
16	Control		0.00001	c.c.	Died, 40 hrs.
17	Control		0.000001	c.c.	Died, 48 hrs.

<sup>2</sup> This indicates the ninety-sixth passage through animals with a single passage on culture media.

TABLE III.

*C. Duration of Protection Afforded by Antipneumococcus Serum and Its Precipitate.*

In this experiment two series of mice were given, respectively, whole serum and dissolved precipitate in a dose of 0.2 c.c. subcutaneously, and inoculated subsequently at intervals with suitable controls.

Series *A*, on Dec. 15, 1914, was given 0.2 c.c. of diluted serum in saline (volume 0.5 c.c.) subcutaneously.

Series *B*, on the same date, was given 0.2 c.c. of dissolved precipitate from serum (0.2 in 0.5 c.c.) subcutaneously.

Mouse.	Interval.	Dose of culture.		Result.
A-1	4 dys.	0.1	c.c.	Died, 14 hrs.
A-2	4 dys.	0.01	c.c.	Died, 18 hrs.
A-3	4 dys.	0.001	c.c. <sup>3</sup>	Survived.
A-4	8 dys.	0.01	c.c.	Died, 20 hrs.
A-5	8 dys.	0.001	c.c.	Died, 10 hrs.
A-6	8 dys.	0.0001	c.c. <sup>4</sup>	Survived.
A-7	10 dys.	0.0001	c.c.	Survived.
A-8	10 dys.	0.00001	c.c. <sup>5</sup>	Survived.
B-1	4 dys.	0.1	c.c.	Died, 18 hrs.
B-2	4 dys.	0.01	c.c.	Died, 18 hrs.
B-3	4 dys.	0.001	c.c. <sup>3</sup>	Survived.
B-4	8 dys.	0.01	c.c.	Died, 10 hrs.
B-5	8 dys.	0.001	c.c.	Died, 10 hrs.
B-6	8 dys.	0.0001	c.c. <sup>4</sup>	Died, 4 dys.
B-7	10 dys.	0.001	c.c.	Died, 24 hrs.
B-8	10 dys.	0.0001	c.c.	Died, 30 hrs.
B-9	10 dys.	0.00001	c.c. <sup>5</sup>	Died, 42 hrs.

The first two sections of this experiment (*A* and *B*) show that the precipitate derived by adding an extract of pneumococcus to anti-pneumococcus serum may contain as much protective power against pneumococcus infection in mice as the original serum from which it is derived. It is active whether employed in its original precipitated condition or dissolved in a small amount of alkali. In this experiment the duration of the immunity passively conferred by precipitate was not so great as when serum was used. This failure in durability, however, may well have been due to the fact that the solution of

<sup>3</sup> Controls with doses of 0.00001 and 0.000001 c.c. died in 40 hrs.

<sup>4</sup> Controls with doses of 0.00001 and 0.000001 c.c. died in 20 and 48 hrs.

<sup>5</sup> Controls with doses of 0.00001 c.c. died in 42 hrs.

precipitate in an alkali is by no means without harmful effect on its protective power. The amount of alkali needed to dissolve the precipitate has varied not only with the apparent opacity of the precipitate but also with its age, freshly formed precipitates being more readily soluble than older ones. At all events the addition of the alkali has frequently robbed the precipitate of its protective properties, and it has likewise a harmful effect on the original serum. A solution of the precipitate seemed desirable in view of its possible application by intravenous injection in human cases of pneumonia. It is questionable, however, if the precipitate itself would be dangerous to employ; we have given considerable amounts to rabbits intravenously without ill effect.

It seemed to be of importance, when employing the original precipitate in protection against intraperitoneal infection in mice, to show that the protective effect is not in some measure due to a possible mechanical effect of the precipitate rather than to specific antibodies. This would seem to be ruled out in the following experiment, in which a precipitate from a serum of type I was employed mixed with cultures of pneumococci of both type I and type II.

*Experiment 2.*—Protective value of precipitate I.<sup>6</sup> The dried culture in this experiment differs from the preceding antigen in that it was precipitated by alcohol directly from the bouillon culture without washing. An extract was made in carbolated saline in dilution of 1 mgm. to 1 c.c. This antigen solution was added in doses of 50 and 40 c.c., with 12 hours' interval, to 100 c.c. of serum of type I, lot 3. Precipitates were produced on each addition, which were then washed and suspended in one-fifth the original volume. Not all the precipitate was removed from the treated serum, as could be shown by further addition of antigen. A Kjeldahl determination of the precipitate diluted to original volume gave a protein content of 0.108 per cent.

The protective values of the precipitate concentrated five times, of the precipitate at original volume, and of the concentrated precipitate dissolved in sodium hydrate,<sup>6</sup> are compared with the original serum. Mixtures of the various fluids were made with the culture dilutions, each in a volume of 0.5 c.c., and injected intraperitoneally in mice (table IV).

<sup>6</sup> 0.3 c.c. of  $\frac{1}{T}$  sodium hydrate was used to dissolve 5 c.c. of five times concentrated precipitate.

TABLE IV.

Mouse.	Protective fluid.	Culture.	Result.
		Pneumococcus, type I, 100-4	
1	Original serum 0.2 c.c.	0.1 c.c.	Survived.
2	Original serum 0.2 c.c.	0.01 c.c.	Survived.
3	Original serum 0.2 c.c.	0.001 c.c.	Survived.
4	Precipitate emulsion, original volume 0.2 c.c.	0.1 c.c.	Died, 36 hrs.
5	Precipitate emulsion, original volume 0.2 c.c.	0.01 c.c.	Survived.
6	Precipitate emulsion, original volume 0.2 c.c.	0.001 c.c.	Survived.
7	Precipitate emulsion concentrated 5 times	0.1 c.c.	Survived.
8	Precipitate emulsion concentrated 5 times	0.01 c.c.	Survived.
9	Precipitate emulsion concentrated 5 times	0.001 c.c.	Survived.
10	Precipitate concentrated 5 times dissolved in sodium hydrate	0.1 c.c.	Died, 4 dys.
11	Precipitate concentrated 5 times dissolved in sodium hydrate	0.01 c.c.	Died, 40 hrs.
12	Precipitate concentrated 5 times dissolved in sodium hydrate	0.001 c.c.	Died, 13 hrs.
		Pneumococcus, type II, 34-7 <sup>1</sup>	
13	Precipitate emulsion concentrated 5 times	0.1 c.c.	Died, 15 hrs.
14	Precipitate emulsion concentrated 5 times	0.01 c.c.	Died, 15 hrs.
15	Precipitate emulsion concentrated 5 times	0.001 c.c.	Died, 18 hrs.
16	Precipitate emulsion concentrated 5 times	0.0001 c.c.	Died, 15 hrs.
		Pneumococcus, type I, 100-4	
17	Control	0.00001 c.c.	Died, 32 hrs.
18	Control	0.000001 c.c.	Died, 24 hrs.
		Pneumococcus, type II, 34-7	
19	Control	0.00001 c.c.	Died, 18 hrs.
20	Control	0.000001 c.c.	Died, 14 hrs.

This experiment indicates the protective value of undissolved precipitate emulsion, shows its specificity, and that concentration increases its potency. The deleterious effect of solution in sodium hydrate is also shown.

We are at present engaged in determining other methods of dissolving the precipitate which may have no effect on the protective bodies. Our experiments show further that the agglutinins as well as the protective bodies are brought down with the precipitate. Preliminary experiments indicate that a comparison of the protective value of precipitates with that of the original serum may be made more convincing by employing rabbits instead of mice. It is at least evident that a simultaneous injection of serum or of the precipitate will protect rabbits from an intravenous dose of the pneumococcus that is many times the fatal dose. The precipitates have shown themselves to be as protective as the original serum in these animals, although the end-point of the protective value has not been reached in our experiments to the present time.

TABLE V.

*Protein Content of Antipneumococcus Serum before and after Precipitation, and of Its Precipitates with Indication of Protective Value of Precipitates.*

Precipitate lot.	Protein content.		Exhausted serum.	Protective value of precipitate.
	Original serum.	Antigen.		
I <sup>1</sup>				Protects well.
I <sup>2</sup> 0.34%				Protects well.
I <sup>3</sup> 0.18%	5.76%		7.0 %	Partial protection. <sup>7</sup>
I <sup>4</sup>				Partial protection. <sup>7</sup>
I <sup>5</sup> 0.09%	6.49%	0.015%	7.19%	Partial protection. <sup>7</sup>
I <sup>6</sup> 0.108%				Protects well.
I <sup>7</sup> 0.101%	5.9 %	0.022%	5.07%	Protects well.
II <sup>1</sup> 0.21%	5.9 %		5.90%	Does not protect.
II <sup>4</sup> 0.157%	5.56%	0.022%	5.72%	Protects well.

It remains to discuss the relative protein contents of serum and precipitate. Our results here are somewhat varying and fragmentary, but show that the protein content of the precipitate as compared with serum is surprisingly low. There would, moreover, seem to be no necessarily direct relation between protein content and protective value. We are indebted to Mr. Cullen for the Kjeldahl determina-

<sup>7</sup> The failure to demonstrate good protection in this serum is probably due to the fact that only the dissolved sodium hydrate precipitate was tested.

tions of total nitrogen content on which these figures are based. The estimates were made in duplicate and usually from five cubic centimeters each of a dilution of each fluid.

It will be noted from table V that in all instances of precipitates from antipneumococcus sera of type I, where only partial protection was obtained by the precipitates, the original precipitate emulsions were not tested, but only the precipitates after solution in sodium hydrate which, as has been evidenced, may destroy the protective power in precipitate or in serum.

The average protein content of more or less perfectly protective precipitates is, as will be noted, very low, ranging from 0.09 to 0.34 per cent., or from one-seventieth to one-thirty-second of the amount of protein in the original serum in those instances in which full computation was made. The obvious value of the possible employment of concentrated immune bodies in a solution of such low protein concentration for intravenous injection in human beings is apparent. The avoidance of serum sickness and of the possible formation of antagonistic bodies seems possible by this means. The advantage of this method of concentration of the protective bodies over concentration by chemical precipitation lies not only in the low protein content of the precipitate, but also in the fact that it may be produced rapidly and under conditions of absolute asepsis.

#### CONCLUSIONS.

The addition of a water-clear extract of pneumococci to homologous antiserum produces a voluminous precipitate which carries down with it the agglutinins and practically the totality of the protective bodies against pneumococcus infection in animals. This precipitate when washed and resuspended in saline solution to the original volume of serum protects as well as the whole serum. The protein content of such solutions has varied from 0.09 to 0.34 per cent., as contrasted with about 6 per cent. in the original serum. The solution of this precipitate is not necessary to insure protection, and when produced by dilute alkali (sodium hydrate) frequently destroys the immune bodies.

In conclusion the senior author wishes to express his appreciation of the courtesy which Dr. Cole has shown in placing the facilities of the hospital at his disposal for this work.

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# THE FACTORS OF COAGULATION IN THE EXPERIMENTAL APLASTIC ANEMIA OF BENZOL POISONING, WITH SPECIAL REFERENCE TO THE ORIGIN OF PROTHROMBIN.\*

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## INTRODUCTION.

In the course of a study (1) on the factors of coagulation in a case of aplastic anemia we were impressed with the analogy existing between the symptom-complex in this disease and that observed by Selling (2, 3) in three cases of benzol poisoning. The chief clinical features presented by Selling's three patients were: first, purpura hemorrhagica, with typical skin lesions, bleeding from the mucous membranes, and retinal hemorrhages; second, a blood picture characterized by a leucopenia of a marked grade, a striking reduction in the blood platelets, and anemia. Pathologically these patients showed well marked aplasia of the bone marrow. A study of his three patients induced Selling to try to produce in animals aplastic anemia with benzol. This he accomplished successfully in rabbits and showed that benzol is a powerful leucotoxic agent, acting chiefly upon the bone marrow, although producing changes of some degree also in other hematopoietic organs.

Workers who have studied the blood in benzol poisoning have concerned themselves thus far chiefly with the formed elements. Selling (3) found both a reduction in the red corpuscles and a striking diminution in the leucocytes, with an almost total disappearance of these elements in the blood of fatally poisoned animals. In addition, Duke (4) found a marked drop in the platelet count. According to Duke, a sufficiently low reduction in blood plates will lead to the prolonged bleeding time noted in benzol intoxication. Duke's studies included also occasional determinations of the blood fibrinogen; but we have been unable to find any complete and systematic studies of the factors of coagulation in experimental conditions associated with aplasia of the bone marrow.

The present series of experiments was undertaken with the hope of learning something about the factors of coagulation in benzol poisoning, but as the work progressed it seemed to give additional information concerning the relation of the bone marrow to the origin of the factors of coagulation studied by us; namely, prothrombin, antithrombin, and fibrinogen.

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Our observations have shown that the clinical symptoms of an animal poisoned with benzol, as far as the hemorrhagic features are concerned, give only slight indication of the profound changes which this myeloid tissue poison produces in the blood of such animals. The latter do not, as a rule, exhibit the usual hemophilic tendency with which we are familiar,—delayed bleeding time, purpura, bleeding from the gums and mucous membranes, etc. Very occasionally one meets with prolonged bleeding from cuts and ear pricks, which, according to the recent observation of Duke (5), occurs only when the plates are reduced to a dangerously low level, 5,000 to 15,000 per cubic millimeter. With the exception of a few instances in which the blood, at autopsy, was found to remain fluid for a long time, no striking departure from normal clotting was noted. A study of the blood showed, however, that striking changes had resulted both in the formed elements and in the prothrombin content, although such changes were not of a degree sufficiently marked to produce the clinical picture of hemorrhagic disease.

As far as the effect of benzol upon the formed elements is concerned, our experiments confirm the work of Selling and of Duke. Following the administration of benzol, there is a rapid disappearance of the white cells from the peripheral circulation. We have observed also an initial rise in some instances (experiments B, F, G, and H). The red blood corpuscles are much less affected than the white. In a few experiments (B, E, and G) the count remained unaltered, whereas in the majority of observations the red cell count was reduced by 50 per cent. or more.

The platelets showed usually the same general reduction in number, though to a less extent. We were impressed with the observation that the blood plates may remain at a high level at a time when the white cells have almost entirely disappeared from the circulation. In only one instance were we able to reduce the number of platelets so low as to reproduce the symptoms of hemorrhagic disease. Such experiments suggest the possibility that the megakaryocytes of the bone marrow either regenerate very rapidly or else are more resistant to the toxic action of benzol than are the forerunners of the polymorphonuclear leucocytes and erythrocytes.<sup>1</sup> This view,

<sup>1</sup> According to Selling's work (*loc. cit.* (3), p. 53), the polymorphonuclear

if correct, would help to explain the difficulty encountered in reproducing by benzol injections the features to which attention has been called.

Besides the formed elements, we have followed the factors of coagulation in the majority of our animals. These studies showed that benzol exerts an important influence in reducing the circulating prothrombin and that this substance is dependent for its production, in part at least, upon bone marrow activity. Two of the other factors of blood clotting, antithrombin and fibrinogen, were also followed in the majority of the experiments. These were found to fluctuate little from the normal.

Our results are in harmony with the recent proof that blood plates contain prothrombin. They give additional evidence in support of the view that fluctuations in the amount of prothrombin may be produced by substances which affect the number of platelets, and that any toxin which produces a reduction in their number will simultaneously cause a diminution in the available prothrombin of the circulating blood. Our knowledge, moreover, of the origin of blood platelets from the megakaryocytes (6) of the bone marrow emphasizes the importance of this tissue in maintaining the normal prothrombin equilibrium of the blood. Although the marrow plays this important part in the elaboration of prothrombin, no definite parallelism could be established between the extent of bone marrow injury, the number of blood plates, and the relative amounts of prothrombin. This would suggest that it is not possible to create a dangerous prothrombin deficiency by a reduction in the number of blood platelets alone, and that some other tissue or tissues play some part in prothrombin formation.

It will be seen, too, that the marrow in no way participates in the production of antithrombin and fibrinogen, for we did not observe any changes in these substances in the blood of animals with aplastic marrow.

#### METHODS.

*Experimental Benzol Poisoning.*—The technique employed by us for producing aplasia of the bone marrow was the same as that used amphophils in the marrow are more resistant than large lymphocytes, myelocytes, erythroblasts, and giant-cells.

by Selling. Because of the knowledge of the pathological lesions produced by benzol in the rabbit, gained from the fundamental observations of this worker, these animals were chosen for our experiments.

Injections were made subcutaneously with chemically pure benzol. The average dose injected was two cubic centimeters of benzol daily per kilogram of body weight, although smaller doses were used when the animals began to show signs of marked intoxication. Sudden death may follow the injection of too large doses. The acute symptoms accompanying such sudden deaths are referable chiefly to the central nervous system, sufficient time not having been allowed for the development of the myelotoxic effect of this poison. It is essential, therefore, to proceed slowly with smaller doses in order to produce the chronic type of poisoning which results in the typical changes in the hematopoietic organs.

We have found certain animals quite resistant to the poison. In some instances, many injections were necessary in order to produce the desired effect. Such resistance, however, is not the usual rule. Most animals succumb after ten doses, and at autopsy show fairly well marked aplasia of the marrow. It was our original plan to bring about an aplasia of the bone marrow, after which regeneration was to be permitted to occur, and to follow the fluctuations of the prothrombin as the animals were returning to a normal state. It was difficult, however, to obtain such an ascending series because repeated injections were necessary to produce demonstrable changes in the prothrombin content of the blood and these usually resulted fatally.

Variations in the white cell count and in the number of blood platelets give a fair index of the condition of the bone marrow, the counts dropping as the aplasia becomes marked. Selling<sup>2</sup> has pointed out that when the number of white cells has reached as low a level as 200 to 800 cells per cubic millimeter, any additional injections usually prove fatal. This has been our experience also.

Thus far we have been unable to reduce the blood platelets to a dangerously low level. In only three experiments did the number of platelets fall below 100,000, and in only one instance to 30,000. This observation may be of interest in connection with a point to

<sup>2</sup> Selling, *loc. cit.* (3), p. 11.

which attention will be directed later, that it is also impossible by this method to diminish sufficiently the prothrombin of the circulating blood so as to render these animals hemophilic.

*Obtaining Blood.*—The problem of obtaining specimens of blood from rabbits with the precautions which are necessary for this work was solved by resorting to cardiocentesis with a graduated Luer syringe. Before introducing the needle the interior of the syringe was coated with a thin layer of a mixture of white petrolatum 25 parts and ether 300 parts, and the needle was filled with salt solution. With a little practice it is possible to obtain blood in this way after one attempt. This precaution is important, since repeated thrusts of the needle into the heart may traumatize its muscle, setting free neutralizing tissue juices, and frequently producing a hemo-pericardium which may embarrass the heart's action and cause sudden death. By the exercise of skill it is possible to repeat cardiocentesis many times without endangering the life of the animal.

For our purposes ten cubic centimeters of blood sufficed. This was immediately emptied into a centrifuge tube containing two cubic centimeters of a 1 per cent. solution of sodium oxalate (made up in 0.9 per cent. solution of sodium chloride). After being thoroughly mixed the specimen was centrifugalized at a constant speed (a definite number of revolutions on our centrifuge) for ten minutes. The plasma, which in rabbits is usually watery or opalescent, due to a slight lipemia, was pipetted off and examined for the various factors of coagulation, according to the methods of Howell (7, 8).

*Enumeration of Formed Elements.*—Of all the methods proposed for the enumeration of blood platelets, that of Wright and Kinnicutt (9) appears to have given the most reliable results in the hands of recent workers. As used by Duke (5) this method has given constant results and was the one we used.

*Quantitation of Prothrombin, Antithrombin, and Fibrinogen.*—In the writings of Howell and his coworkers will be found a comprehensive description of the methods introduced by him for determining the amount of prothrombin and antithrombin in blood. In a recent paper the authors have already commented upon the various methods and their usefulness. For purposes of clearness, however, we wish to mention them briefly at this time, particularly with refer-

ence to certain precautions to be followed in carrying out the prothrombin test.

*Prothrombin.*—As yet no good method is available for the isolation and absolute quantitation of prothrombin in blood plasma. An idea of the relative amount or relative strength of the prothrombin in the blood may be obtained, however, by a simple method devised by Howell.<sup>3</sup> This depends upon the observation that the act of oxalating, that is, the decalcifying of the solution, intensifies the activation of prothrombin to thrombin by a subsequent calcification. This results apparently in a greater production of available thrombin for interaction with the fibrinogen of the plasma.

In practice the reaction is carried out as follows: To a series of tubes containing a constant amount of oxalated plasma is added dilute calcium chloride in varying amounts. Coagulation will result and the time of coagulation, as measured by the invertibility of the clot, will be shortest in the tube containing the optimum amount of calcium. Tested by this method, the clotting time of normal rabbit plasma averages about ten minutes.

To insure uniform results, it is necessary to take two precautions: first, the employment of an equal quantity of each reagent; second, centrifugalization of the plasma at a constant speed and for a constant number of minutes in all comparable observations. That the speed of this prothrombin reaction can be influenced by centrifugalization was shown by Lee and Vincent (10) and again emphasized by Howell in a recent publication (11). This was shown to depend upon variations in the thromboplastin content of plasma centrifugalized for different periods of time. Failure to observe these two points may lead to erroneous results in experiments where no wide differences exist between the quantity of prothrombin present in normal and in pathological blood.

*Antithrombin.*—The method of demonstrating the anticoagulating action of normal and pathological plasmas has been so clearly described in recent papers that only brief mention of the technique need be considered here. The test plasma is heated slowly to 60° C. and then centrifugalized to remove the fibrinogen and prothrombin. A drop of this plasma containing the antithrombin is then added to

<sup>3</sup> Howell, *loc. cit.* (7), p. 78.

known amounts of thrombin solution. After a short period of incubation, fibrinogen solution is added. The addition of the antithrombin delays or inhibits completely the action of thrombin on fibrinogen. In tests in which the thrombin is nearly neutralized by the antithrombin, it is very difficult to ascertain when clotting has occurred, since under such circumstances the clot forms in several stages. For purposes of uniformity we have adopted as the end-point of this reaction the first appearance of a delicate or filmy clot. In clinical tests the relative amount of antithrombin in the oxalated plasma of the patient is compared always with a similar specimen from a normal person; in our animal experiments the initial observation made upon the healthy animal always under exactly the same conditions, and with the same thrombin and fibrinogen solutions where possible, served as the control. Reference to one of the protocols given below will help to make clear the method of making the comparisons.

*Fibrinogen.*—The heat coagulation method of determining fibrinogen has also been described many times in recent communications. For details of this method reference should be made to papers by Whipple and Hurwitz (12) and by Whipple (13).

#### EXPERIMENTAL DATA.

With the exception of one or two experiments, to which attention will be directed later, the observations may be divided into two main groups: first, those in which the animals showed great susceptibility to the poison, as indicated clinically by a great drop in the formed elements, and pathologically by an aplasia of the bone marrow of varying degree; second, those in which less striking reduction in the formed elements, particularly in the blood platelets, could be produced, and in which autopsy revealed either a less well marked aplasia or in a great many instances a marrow in which the regenerative process was keeping pace with the destructive changes. In the first group a diminution in the circulating prothrombin was demonstrable; whereas in the second group little or no change from the normal was observed.

In all, about fifteen complete experiments were performed. Of these the following two, one from each group, will be summarized,

since they are fairly complete and quite representative of all the other experiments.

TABLE I.  
*Rabbit P.*

Date.	Weight in gm.	Dose of benzol in c.c.	Formed elements.			Factors of coagulation.				
			Platelets.	W. B. C.	R. B. C.	Prothrombin.		Antithrombin. <sup>4</sup>		Fibrinogen in gm. per 100 c.c.
						CaCl <sub>2</sub>	Min.	Thrombin.	Min.	
Nov. 11	1,400	2	920,000	7,800	5,200,000	1	11	3	15	0.719
						2	15	4	5	
						3	15	5	5	
						4	15			
Nov. 12-15	1,350	6	480,000	3,200	4,700,000					0.656
Nov. 15		2				1	40	3	No clot in 60	
						2	31	4	6	
						3	30	5	8	
Nov. 16-18	1,175	4	185,000	1,900	3,710,000	4	38			0.684
Nov. 19		1				1	60	3	23	
						2	60	4	7	
						3	60	5	5	
Nov. 20-24	1,100	8	128,000	500	1,810,000	4	60			0.638
Nov. 25						1	50	3	5	
						2	55	4	12	
						3	65	5	5	
						4	95			

PROTOCOL.

*Rabbit P.*—(Table I.) Nov. 11. Young brown male; weight 1,400 gm. Red blood count 5,200,000; white blood count 7,800; platelets 920,000; fibrinogen 0.719 gm. per 100 c.c. of plasma.

*Prothrombin Test.*

Oxalated plasma.	CaCl <sub>2</sub> , 1 per cent.	Coagulation.
5 drops	1 drop	11 min.
5 drops	2 drops	15 min.
5 drops	3 drops	15 min.
5 drops	4 drops	15 min.

*Antithrombin Test.*<sup>5</sup>

Heated plasma.	Thrombin.	Time interval.	Fibrinogen solution.	Coagulation.
1 drop	3 drops	15 min.	7 drops	15 min.
1 drop	4 drops	15 min.	7 drops	5 min.
1 drop	5 drops	15 min.	7 drops	5 min.

Benzol 2 c.c. given subcutaneously.

<sup>4</sup> Control solutions of fibrinogen and thrombin clotted in from two to four minutes.

<sup>5</sup> Control 2.5 to 3 min.



Nov. 12, 13, and 14. On each of these dates 2 c.c. of benzol were injected. Following the injections the animal became dull, drowsy, and inactive.

Nov. 15. Weight 1,350 gm.; red blood count 4,700,000; white blood count 3,200; platelets 480,000; fibrinogen 0.656 gm. per 100 c.c. of plasma.

*Prothrombin Test.*

Oxalated plasma.	CaCl <sub>2</sub> , 1 per cent.	Coagulation.
5 drops	1 drop	40 min.
5 drops	2 drops	31 min.
5 drops	3 drops	30 min.
5 drops	4 drops	38 min.

*Antithrombin Test.<sup>6</sup>*

Heated plasma.	Thrombin.	Time interval.	Fibrinogen solution.	Coagulation.
1 drop	3 drops	15 min.	7 drops	No clot in 1 hr.
1 drop	4 drops	15 min.	7 drops	6 min.
1 drop	5 drops	15 min.	7 drops	8 min.

Nov. 16, 17, and 18. Daily injections of benzol amounting to 4 c.c. Animal is beginning to look very ill; very weak; does not eat. No paralysis of legs noted.

Nov. 19. Weight 1,175 gm. Red blood count 3,710,000; white blood count 1,900; platelets 185,000; fibrinogen 0.684 gm. per 100 c.c. of plasma.

*Prothrombin Test.*

Oxalated plasma.	CaCl <sub>2</sub> , 1 per cent.	Coagulation.
5 drops	1 drop	60 min.
5 drops	2 drops	60 min.
5 drops	3 drops	60 min.
5 drops	4 drops	60 min.

*Antithrombin Test.<sup>7</sup>*

Heated plasma.	Thrombin.	Time interval.	Fibrinogen solution.	Coagulation.
1 drop	3 drops	15 min.	7 drops	23 min.
1 drop	4 drops	15 min.	7 drops	7 min.
1 drop	5 drops	15 min.	7 drops	7 min.

Benzol 1 c.c. given subcutaneously.

Nov. 20, 21, 22, 23, and 24. Benzol 8 c.c. injected during these five days. Animal becoming very weak and emaciated.

Nov. 25. Weight 1,100 gm. Red blood count 1,810,000; white blood count 500; platelets 128,000; fibrinogen 0.638 gm. per 100 c.c. of plasma.

*Prothrombin Test.*

Oxalated plasma.	CaCl <sub>2</sub> , 1 per cent.	Coagulation.
5 drops	1 drop	50 min.
5 drops	2 drops	55 min.
5 drops	3 drops	65 min.
5 drops	4 drops	95 min.

<sup>6</sup> Control 2.5 min.

<sup>7</sup> Control 3.5 min.

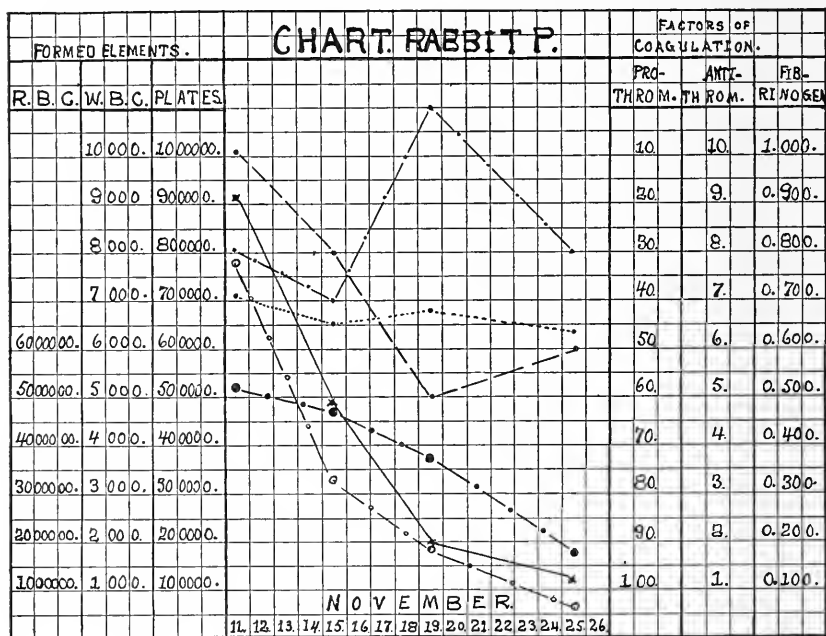
*Antithrombin Test.*<sup>8</sup>

Heated plasma.	Thrombin.	Time interval.	Fibrinogen solution.	Coagulation.
1 drop	3 drops	15 min.	7 drops	5 min.
1 drop	4 drops	15 min.	7 drops	12 min.
1 drop	5 drops	15 min.	7 drops	5 min.

Nov. 26. Animal killed and autopsied at once. Serous cavities were all normal except for a small organized clot in the pericardial sac. The heart showed many small scars covered with fibrin at the points where the cardiac muscle was pierced in obtaining blood. No gross changes were demonstrable in any of the other organs. Bone marrow obtained from the middle of the femur was mottled brown, of fair consistency, with small pin-point hemorrhages.

Microscopical sections showed an aplastic marrow in the stage of fairly advanced regeneration. In some parts of the section the aplasia was found to be fairly complete, but superimposed upon the edematous aplastic reticulum there were groups and clumps of parenchymal cells; these consisted of granulocytes and normoblasts. Megakaryocytes were present in fairly large numbers.

Reference to the protocol and to table I and text-figure 1 will



TEXT-FIG. 1. Formed elements: heavy dots and dashes = red blood corpuscles; open circles and dashes = white blood corpuscles; solid line = blood platelets. Factors of coagulation: broken line = prothrombin; small dots and dashes = antithrombin; dotted line = fibrinogen.

<sup>8</sup> Control 3 min.

show the effect of benzol poisoning upon both the formed elements and the factors of coagulation in this particular animal. The observations, in this instance, were carried on over a period of two weeks. At the outset the animal was healthy, active, and weighed about 1.5 kilograms. Examinations of the formed elements and the factors of coagulation made at the beginning of the experiment (November 11) showed normal figures. It may be said, however, that the platelet count represents the high limit of normal, and that this is true also of the blood fibrinogen. Benzol injections were given daily, the average dose being two cubic centimeters. Only after four such injections were given did the animal begin to manifest any signs of intoxication. Studies of the blood at this time showed about 50 per cent. reduction in the blood platelets, a similar reduction in the white cells, a moderate grade of anemia, a prothrombin content (as measured by the coagulation time of the recalcified plasma) one-third of the control, an unaltered antithrombin content, and a very slight reduction in the blood fibrinogen.

As the benzol injections were continued, the animal began to exhibit more marked loss of weight, emaciation, anorexia, and dullness. No tendency to bleed was observed, however; but, as indicated in the protocol, both the formed elements and the prothrombin showed a still greater reduction at this time. At the end of two weeks, after twenty-three cubic centimeters of benzol had been injected, a final study of the blood was made. The platelets had fallen to 13 per cent. of the normal, and the prothrombin diminished to such an extent that the coagulation time was six times the control. The antithrombin and fibrinogen values were found to show normal variations.

For purposes of brevity the protocol for experiment G is not reproduced here. The results obtained on this animal are recorded in table II. In this experiment, an example of the second group, determinations of the formed elements and coagulation factors were made over a period of six weeks (September 1 to October 11). At the beginning of the experiment all the formed elements and the prothrombin showed normal values. Unfortunately the antithrombin and fibrinogen were not followed until several weeks after the benzol injections were commenced. Throughout the course of the experi-

TABLE II.

*Rabbit G.*

Date.	Weight in gm.	Dose of benzol in c.c.	Formed elements.			Factors of coagulation.				
			Platelets.	W. B. C.	R. B. C.	Prothrombin.		Antithrombin.		Fibrino- gen in gm. per 100 c.c.
						CaCl <sub>2</sub>	Min.	Throm- bin.	Min.	
Sept. 1	2,200			4,500	4,400,000	1 2 3 4	0 9 10 11			
Sept. 2-5		6	840,000							
Sept. 5	2,040		Sept. 2 520,000	2,400	5,640,000	1 2 3 4	0 17 14 45			
Sept. 7-9		4								
Sept. 9	1,800		612,000	5,600	4,430,000	1 2 3 4	25 22 25 17			
Sept. 11		2								
Sept. 13	1,640		216,000	200	5,670,000	1 2 3 4	18 15 19 19			0.925
Sept. 14		2								
Sept. 16	2,085	2	240,000	1,500	5,100,000	1 2 3 4	12 9 18 9	3 4 5	45 10 5	
Sept. 17-18	2,400	2	144,800	1,800	3,470,000	1	Lost	3	25	0.900
Sept. 19	2,085		180,000	1,200	2,880,000	2 3 4	18 18 18	4 5	7 7	
Sept. 22-26	1,890	4								
Oct. 2	1,830	2		1,400	3,730,000	1 2 3 4	13 13 12 13	3 4 5	40 27 10	0.252
Oct. 3-4		4								
Oct. 5	1,795	2	196,000	1,460	3,780,000	1  2 3 4	8  6 8 8	3  4 5	No ob- serva- tion 20 20	0.273
Oct. 6-11	1,425	16	276,000	1,400	4,230,000					

ment, this animal showed unusual resistance to benzol poisoning, and in spite of the development of transient palsy of the right leg, remained fairly healthy. As the intoxication progressed, the plate-

lets reached as low a level as 180,000, the white cells 1,200, and the red cells 2,800,000. Determinations of the coagulation time of the recalcified plasma during this period showed variations between nine and eighteen minutes, the time becoming less than normal (six minutes) toward the end of the experiment. This shows that the prothrombin content of the blood remained normal in spite of the long period of intoxication and the quite well marked drop in the platelets.

The first antithrombin determinations were made two weeks after the experiment began; this factor was followed subsequently, and at the completion of the experiment no change was noted from the initial determination.

The fibrinogen in the blood fell precipitously during the last three weeks of the experiment. From an initial high level of 0.900 of a gram it fell to 0.273 of a gram. At the time of the last determination, the animal was much emaciated, having lost about 400 grams in weight. This is the only instance in which we have observed such an extensive reduction in fibrinogen following benzol poisoning. We are not prepared to explain this, unless it is due to the gradual wasting and emaciation which developed during the long period of intoxication.

At autopsy we were interested to find that this animal showed evidences of a wide-spread regeneration of the bone marrow. Zones of aplasia still were present, but these were small. Parenchymal elements were found scattered diffusely in long strands throughout the edematous reticulum. Granulocytes and normoblasts were present in abundance, and the megakaryocytes were also found in considerable number. The relatively high platelet count, in spite of repeated benzol injections, is in keeping with such a regenerating marrow.

#### SUMMARY OF RESULTS.

*Formed Elements.*—By referring to tables III and IV, it will be seen that all the formed elements are reduced materially in the blood of benzolized rabbits, although the leucotoxic action is the most pronounced. In normal rabbits the average leucocyte count is 7,820 with variations between 11,000 and 4,200. With few exceptions benzol injections cause a striking drop in the count. In our series the white cells dropped to an average level of 1,390, with varia-

TABLE III.  
Summary. Prothrombin Diminished. Antithrombin and Fibrinogen Normal.

Date.	Rabbit.	Weight in gm.	Total dose of benzol in c.c.	Formed elements.			Factors of coagulation. <sup>9</sup>			
				Platelets.	W. B. C.	R. B. C.	Prothrombin per min.	Antithrombin per min.	Fibrinogen in gm. per 100 c.c.	
Aug. 15 Aug. 24	B		18		4,200 0	5,400,000 5,480,000	6 35			Bone marrow.
Aug. 31 Sept. 5 Sept. 10	E	1,460 1,420 1,200	8	760,000 520,000 148,000	8,400 3,100 900	4,960,000 4,430,000 3,930,000	9 14 310			Well marked aplasia. Reticulum aplastic and hyperemic; small nests of erythroblasts; megakaryocytes fairly numerous.
Oct. 2 Oct. 5 Oct. 7 Oct. 10	K	1,380 1,305 1,300 1,160	12	408,000 502,000 336,000	8,400 11,200 20,800	5,980,000 3,980,000 4,200,000 2,130,000	8 8 18 34	27 23 15	0.387 0.679 0.639 0.899	Advanced aplasia. Edema and hyperemia of reticulum; practically no parenchymal elements remaining, excepting fixed tissue cells, lymphocytes, and polyblasts.
Oct. 2 Oct. 5 Oct. 11	L	1,620 1,550 1,205	18	720,000 364,000	6,400 2,200	4,200,000 3,800,000 3,000,000	13 10 24	31 35 19	0.543 0.702	Well marked aplasia (compare rabbit B).
Nov. 11 Nov. 15 Nov. 19 Nov. 25	N	1,300 1,300 1,122 910	23	780,000 320,000 234,000 64,800	10,400 3,000 1,400 800	4,100,000 4,070,000 2,800,000 2,300,000	10 19 39 70	12 9 11 17	Lost 0.532 0.524 1.200	Advanced aplasia. Almost complete disappearance of parenchymal elements (compare rabbits E and L).
Nov. 11 Nov. 19 Nov. 25	O	1,460 1,345 1,170	23	620,000 242,800 31,200	9,200 3,200 900	5,800,000 3,980,000 2,100,000	15 39 45	8 14 8	0.510 0.438 0.422	Advanced aplasia. Microscopic picture identical with that of rabbits L and N.
Nov. 11 Nov. 15 Nov. 19 Nov. 25	P	1,400 1,350 1,175 1,100	23	920,000 480,000 185,000 128,000	7,800 3,200 1,900 500	5,200,000 4,700,000 3,710,000 1,810,000	11 30 60 50	8 7 11 8	0.719 0.656 0.684 0.638	Aplastic marrow with early regeneration. Areas of complete aplasia with groups and islands of parenchymal cells, erythroblasts, and granulocytes. Megakaryocytes numerous but poorly preserved.
Nov. 11 Nov. 15 Nov. 19 Nov. 25	Q	1,640 1,510 1,440 1,470	23	530,000 524,000 120,000 76,000	8,800 4,700 4,000 980	5,400,000 4,200,000 4,200,000 2,800,000	12 16 30 50	8 9 11 9	0.226 0.368 0.321	Aplasia with well advanced regeneration. Long strands of parenchymal cells; megakaryocytes, both young and adult forms, in large numbers.
Dec. 2 Dec. 10 Dec. 12 <sup>10</sup>	R	1,930 1,600	14	700,000 280,000	11,000 2,100	4,200,000 4,400,000	9 25 75	19 16 7	0.852 0.708	Advanced aplasia. Only fixed tissue cells, lymphocytes, and polyblasts present.

<sup>9</sup> The figures in the prothrombin columns represent the coagulation time in minutes of the tube containing the optimum amount of calcium; the figures in the antithrombin columns represent the average clotting time of the tubes containing 3, 4, and 5 drops of thrombin.

<sup>10</sup> Blood obtained in the usual manner five minutes after death.

TABLE IV.  
Summary. Prothrombin, Antithrombin, and Fibrinogen Normal.

Date.	Rabbit.	Weight in gm.	Dose of benzol in c.c.	Formed elements.			Factors of coagulation.			Bone marrow.
				Platelets.	W. B. C.	R. B. C.	Pro- throm- bin per min.	Anti- throm- bin per min.	Fibrino- gen in gm. per 100 c.c.	
Sept. 1	F	2,125	15	600,000	6,600	5,110,000	8			Aplasia. <sup>11</sup> Marked edema of reticulum; capillaries filled with red cells; clumps of erythroblasts and polyblasts; megakaryocytes numerous.
Sept. 5		2,110		560,000	3,300	5,640,000	17			
Sept. 11		2,010		312,000	266	3,760,000	8			
Sept. 1	G	2,200	46	840,000	4,500	4,480,000	9			Marrow in state of active regeneration. Large islands of granulocytes separated by small areas of aplastic reticulum; numerous islands of normoblasts inside and outside of capillaries; megakaryocytes fairly abundant.
Sept. 5		2,040		520,000	2,400	5,640,000	14			
Sept. 9		1,800		612,000	5,600	4,430,000	17			
Sept. 13		1,640		216,000	200	5,670,000	18	0.925		
Sept. 16		2,080		240,000	1,500	5,100,000	9	20		
Sept. 19		2,025		180,000	1,200	2,800,000	18	13	0.900	
Oct. 2		1,830			1,400	3,730,000	13	25	0.252	
Oct. 5	H	1,795		196,000	1,460	3,780,000	6	20	0.273	Hyperplasia of marrow. Parenchymal cells in dense masses and well preserved.
Sept. 14		1,600		744,000	6,600	6,400,000	11	10	0.716	
Sept. 16		1,500	4	254,000	10,200	4,550,000	15	12		
Sept. 14	I	2,375	6	460,000	7,800	5,410,000	13	9	0.123	No sections.
Sept. 19		2,205		280,000	2,800	5,090,000	16	15	0.240	
Dec. 2		1,510		650,000	8,000	4,900,000	10	7	0.432	
Dec. 10	U	1,200	14	570,000	3,800	3,700,000	20	11	0.768	Regenerating marrow (compare rabbit G).
Dec. 2	T	1,730	42	840,000	9,200	5,600,000	9	8	0.252	Hyperplasia of marrow (compare rabbit H). Normal architecture preserved; no areas of aplasia; cellular elements increased in number; granulocytes and erythroblasts well preserved; megakaryocytes numerous, and of young and old type.
Dec. 10		1,600		412,000	6,300	4,900,000	20	6	0.211	
Dec. 14		1,550		253,000	7,200	3,940,000	20	6	0.260	
Jan. 8		1,470		328,000	2,000	2,800,000	9	5	0.728	

<sup>11</sup> Death occurred five days after the last prothrombin determination. On the day before death platelets were 208,000; white blood count 600; and red blood count 2,730,000.

tions between 3,800 and the complete disappearance of leucocytic elements from the circulating stream. The extent of reduction depends upon the susceptibility of the animal. We have records of only two instances in which the intoxication was associated with a leucocytosis. In one of these (experiment K), after an initial fall, the white cells rose rapidly to 20,800; autopsy gave no satisfactory explanation of this leucocytosis. In the second observation (experiment H) the leucocyte count rose forty-eight hours after the first injection. This may be explained by the initial stimulating action of the benzol.

Some reduction in the number of platelets of the circulating blood is invariably the rule. The fall in count, however, is not usually so pronounced, in most instances, as that of the leucocytes. The average platelet count in normal rabbits was found to be 683,400, with variations between 920,000 and 408,000. These averages approximate those recorded by Duke (4) (average 757,000, with variations between 1,200,000 and 380,000). Following benzol inoculations wide variations in the extent of platelet reduction occur. In our series the average number of platelets following benzol injections was 233,800. Only once were we successful in lowering the count to 31,000. This fact explains our inability to produce in these animals hemorrhagic symptoms, such as occur only after more extreme lowering of the platelet count.

Where the platelets and leucocytes were found much diminished in number, the marrow usually showed fairly well marked aplasia. It should be emphasized, however, that destructive changes rarely were noted without signs of regeneration. In some specimens megakaryocytes could hardly be found; whereas in others giant-cells were present in enormous numbers (experiment Q). In the latter case it was apparent that regeneration was going on at a very rapid rate. It is not unlikely that such rapid regenerative phenomena offer some explanation for the difficulty encountered in reducing the blood platelets in rabbits to a dangerously low level.

Although the erythroblastic tissue of the bone marrow suffers, the circulating erythrocytes are injured relatively little. From an average cell count of 5,100,000 the erythrocytes in our series fell to an average level of 3,500,000 following benzol injections. The lowest



reduction in number was obtained in experiment P, in which the red cells were reduced to 1,800,000. As marked an anemia as Selling noted in his clinical cases of benzol poisoning (640,000 and 1,150,000 red cells) apparently cannot be reproduced experimentally in this way.

*Antithrombin.*—Determinations of the amount of antithrombin in the blood were made in twelve of the fifteen experiments. Great care was exercised to keep the technique uniform throughout the series, and in seven of the experiments the same strengths of thrombin and fibrinogen solutions were used, so that these latter are comparable among themselves. In none of the examinations was the variation from the control found to be large. Of the twelve complete records; about six (experiments G, H, O, P, Q, and T) showed an unaltered antithrombin content following benzol inoculations; three (experiments K, L, R) showed a slightly diminished, and three (experiments N, I, and U) a slightly increased amount. The slight diminution or excess observed in some instances depends probably upon the period during which the observation was made. It will be seen from text-figure 1 (rabbit P) that there was an interval during the experiment (November 19) when the antithrombin of the blood was present in excess (compare also experiments G, L, and O). Such fluctuations in the relative amount of antithrombin are due most likely to variations in the supply of tissue juices (thromboplastin), for it will be recalled that during the normal circulation, platelets are continually undergoing dissolution, and that the thromboplastin which they furnish is capable of neutralizing antithrombin.

*Prothrombin.*—The relative amounts of prothrombin, before and after benzol injections, were determined by the method already described. It may be said, in general, that the time of coagulation of the recalcified plasma of normal rabbits was fairly constant, averaging about ten minutes, with variations between six and fifteen minutes. The average coagulation time of such plasma of benzolized rabbits was found to be about fifty minutes, although, as will appear from table III, wide variations of between twenty-four and three hundred and ten minutes were observed. It is of interest that in the latter experiment, which represents an isolated instance where the coagulation time was so markedly prolonged, the marrow

at autopsy showed the most complete aplasia observed in the entire series.

The delayed coagulation of the recalcified plasma of benzolized rabbits may be explained theoretically in one of two ways: it may be due, in the first place, to the presence of an excessive amount of antithrombin; or, in the second place, to a deficiency of prothrombin. In the preceding paragraphs it has been shown, however, that the amount of antithrombin is not altered materially by benzol injections; in fact, there may be relative diminution in the amount, so that the prothrombin deficiency may be even greater than the recorded coagulation time indicates. It is fair to assume, therefore, that the delay in the coagulation time of the recalcified plasma is due to an actual deficiency of prothrombin, and not to an excess of antithrombin.

An examination of table III will show, furthermore, that this prothrombin deficiency does not parallel absolutely the reduction in blood platelets. For instance, certain animals with a much lower count may show a less marked diminution in prothrombin than animals with a higher count (compare experiments E, P, and U with O and Q). Such findings are readily explainable, however, if it is kept in mind that regeneration of the myeloid tissue usually goes hand in hand with aplasia, and that the number of platelets at any given time will depend largely upon the degree of regeneration. This fact helps also to explain why such difficulty is encountered in lowering both the platelets and the prothrombin of the circulating blood to a dangerous point.

No definite parallelism was demonstrable between the prothrombin deficiency and the reduction in leucocytes. The white cells may be much reduced or absent at a time when the prothrombin content is little altered (experiments F and G), or there may be some deficiency in prothrombin associated with a leucocytosis (experiment K). In general, however, a prothrombin deficiency has been found associated with a reduction in the number both of platelets and leucocytes.

*Fibrinogen.*—All the fibrinogen determinations were made by the method of heat coagulation. In every instance the reading was corrected for the oxalate solution used in receiving the blood, so that

the results represent the quantity of fibrinogen in 100 cubic centimeters of actual plasma. Wide fluctuations in the blood fibrinogen of normal rabbits were observed. The average was 0.560 of a gram per 100 cubic centimeters of plasma, but variations between a minimum of 0.123 of a gram, and a maximum of 0.925 of a gram were noted. This is in harmony with Whipple's figures for normal healthy dogs (average 0.466 of a gram; minimum 0.198 of a gram; maximum 0.867 of a gram) (13). In almost all instances repeated injections of benzol (four to forty-two cubic centimeters) caused a rise in the blood fibrinogen. The average of the final readings made from two to thirty days after the initial determination was found to be 0.617 of a gram per 100 cubic centimeters of plasma, but in some individual observations enormous rises were observed (experiments I, K, L, N, Q, T, and U). In about three experiments (O, P, and R) little variation from the normal occurred, and in only one instance (experiment G) was there a striking reduction in the blood fibrinogen. A possible explanation for this drop has been found in the marked loss of weight and extreme cachexia produced in this animal by the benzol inoculations. Similar reductions in the quantity of fibrinogen have been reported by Whipple in human cases of cachexia. The low values in such cases are difficult to explain.

The rise in blood fibrinogen observed in the majority of animals following benzol injections has been observed also in other conditions associated with intoxication.<sup>12</sup> It is possible that benzol may stimulate the fibrinogen-forming organs to over-functional activity and that the fibrinogen may rise above normal at these times. Such an increase is not out of keeping with the gross anatomical and the histological appearance of the liver in benzol poisoning. Aside from a slight degree of parenchymatous change, no other pathological lesion was demonstrable in the liver parenchyma.

THE RELATION OF THE BONE MARROW TO THE ORIGIN OF  
PROTHROMBIN, ANTITHROMBIN, AND FIBRINOGEN.

The experiments and observations already detailed throw some additional light upon this more complex problem of coagulation. In-

<sup>12</sup> Whipple, *loc. cit.* (13), p. 57.

terest in the origin of the various substances concerned in the clotting of blood has stimulated much work among investigators in this field. From time to time different tissues and organs have been made responsible for the elaboration of the factors participating in the process; and, although there are still some workers (14, 15, 16) who ascribe to the liver the important function of forming all the fibrin factors, the majority are agreed that certain of the formed elements of the blood play an important part in the origin of some of these substances. Without entering upon a discussion of the experimental basis for their views, we wish to review briefly some of the work of different observers which is or is not in harmony with our deductions.<sup>13</sup>

*Origin of Prothrombin.*—With the exception of Wooldridge<sup>14</sup> all workers since Alexander Schmidt (17) have recognized the importance of the formed elements of the blood in the elaboration of prothrombin. The view, which originated with Schmidt, that the leucocytes give rise to this fibrin factor has been discredited largely by later work (11, 18). Bizzozero (19) was the first to suggest the platelets as the possible prothrombin formers. To him, too, we owe the knowledge that these elements are independent morphological structures and not degeneration products of leucocytes or erythrocytes. The work of Morawitz (18) has furnished the necessary experimental evidence that the theory of Bizzozero and others was correct. Both Morawitz and more recently Bayne-Jones (20), working in Howell's laboratory, have demonstrated conclusively that aqueous extracts of platelets obtained by differential centrifugalization contain a substance which will clot fibrinogen solutions in the presence of calcium. They have shown further that platelets contain another substance, thromboplastin, so that the disintegration and solution of platelets, when blood is shed, facilitate clotting in two ways: first, by setting free prothrombin; second, by liberating a thromboplastic substance which hastens coagulation by neutralizing the anti-thrombin present normally in circulating blood.

*Origin of Antithrombin.*—It has long been known that the intravenous injection of peptone solutions into animals gives rise in the blood of these animals to a substance which is capable of inhibiting coagulation (Schmidt-Mülheim, 1880). The French School, in particular, has done much experimental work to learn in what organ and by what mechanism this anticoagulation substance is formed. The work of Contjean (21), Delezenne (22), and others has established with certainty that the liver is essential for the formation of this substance, and that antithrombin is not formed if the liver be excluded. There is no direct evidence, however, that this coagulation-inhibiting substance is of the same nature as the antithrombin present normally, in greater or less amount, in

<sup>13</sup> Excellent discussions of this phase of the subject will be found in the papers by Morawitz (prothrombin), Delezenne (antithrombin), and Whipple and Hurwitz and Meek (fibrinogen).

<sup>14</sup> Cited by Morawitz (18).

the blood of animals and man. Their identity is assumed because they both act in a similar way. In this connection it is of interest to note that Whipple (23), in a study of clinical cases, observed fluctuations in antithrombin in the blood of patients suffering from liver disease and in one patient with aplastic anemia. In the latter instance antithrombin was present in excess, although the marrow was completely aplastic. It would appear, therefore, that the reaction of the bone marrow is not a factor in the production of antithrombin. This observation is in harmony with the conclusion deduced from our experiments; namely, that aplasia of the marrow in rabbits produces no appreciable change in the antithrombin of the blood.

*Origin of Fibrinogen.*—An extensive discussion concerning the origin of blood fibrinogen is not needed, as this phase of the subject has been treated fully in several recent papers (13, 24). Suffice it to say that various workers have adduced evidence in support of one or other of the following theories regarding its origin: first, that fibrinogen is formed by the intestine (Mathews (25)); second, that fibrinogen is either produced in the liver or is wholly dependent upon that organ for its production (Doyon (26) and his coworkers, Nolf (27), Whipple and Hurwitz (12), and Meek (24)); third, that fibrinogen is formed by the bone marrow and is perhaps dependent upon the white cells (Müller (28), Morawitz and Rehn (29)).

It would appear that the best experimental evidence is in favor of the second view, although recent studies (Goodpasture (30)) on the regeneration of fibrinogen show that normal fibrinogen production is a result of the combined activity of the liver and the intestine, the latter organ being an important contributing factor in the rapid formation of fibrinogen.

As far as the authors are aware, no direct experimental proof has been produced for or against the view of Müller and of Morawitz and Rehn that the bone marrow is a fibrinogen former. Only a few casual observations are recorded by recent workers upon this function of the marrow. Whipple and Hurwitz<sup>15</sup> noted that with the rapid drop in fibrinogen following chloroform poisoning, there may be a leucocytosis caused by the liver necrosis and repair, and that the bone marrow at such a time may be hyperplastic. Conversely, Whipple,<sup>16</sup> in a study of a clinical case of aplastic anemia, has shown the blood fibrinogen to be normal (0.4032 of a gram), whereas the bone marrow at autopsy was found to be completely aplastic. Similarly in our observations upon experimental aplastic anemia in benzolized rabbits, we have seldom noted low fibrinogen values associated with aplasia of the bone marrow. In almost all our observations the fibrinogen was found either little changed from the normal or somewhat higher than normal. In only one experiment (G) was there any appreciable drop in fibrinogen, and we believe that this has been satisfactorily explained. It seems fair to conclude from such evidence that the bone marrow does not participate in the production of blood fibrinogen.

<sup>15</sup> Whipple and Hurwitz, *loc. cit.*, p. 138.

<sup>16</sup> Whipple, *loc. cit.* (13), p. 59.

## DISCUSSION.

The experimental observations recorded have shown that injury to the myeloid tissue by benzol causes a diminution in the prothrombin content of the blood, and that this tissue must be intimately associated with the elaboration of this substance. Our experiments offer no convincing evidence, however, as to the relative importance of the various formed elements of the marrow in prothrombin production; but the more direct proof given by other workers makes it fairly certain that the platelets and not the leucocytes are the essential elements. Neither Morawitz (18) nor Howell (11) have been able to obtain satisfactory proof that the white cells contain any appreciable amount of prothrombin, whereas both workers have prepared active prothrombin solutions from aqueous extracts of blood platelets. In view of these observations the conclusion appears justified that the blood platelets play more than a mere mechanical part in the process of coagulation. Presumably, during the normal circulation, these elements undergo more or less gradual dissolution and thus help to maintain the prothrombin equilibrium of circulating blood.

Already attention has been directed to the lack of parallelism between the drop in prothrombin and the extent of destruction of myeloid tissue. This finding is in keeping with the experimental and clinical observations of other workers. Pratt (31), for instance, has shown that there is no direct interdependence between the number of platelets and the coagulation time. More recently Duke has emphasized this point by showing that blood in which the platelets are reduced to 10 per cent. of normal by repeated benzol injections may clot at a normal rate. Undoubtedly in such extreme cases the delayed bleeding time must depend upon the mechanical importance of these elements, for it is well known that agglutination of platelets is essential to effective clotting. It follows, therefore, that the platelet count and the circulating prothrombin may be considerably diminished without causing symptoms of hemophilia.

On the whole, the conservative conclusion would seem to be that the maintenance of the prothrombin equilibrium of the blood depends only in part upon the blood platelets. Besides the facts already mentioned, there is other evidence in support of this view. It is well established, for instance, that the platelet count may be

perfectly normal in hemophilia in which the coagulation time may be markedly prolonged in consequence of a deficiency in the amount of the contained prothrombin (7). Furthermore, in a recent publication, Howell (11) has shown that prothrombin is present in solution in the circulating lymph, although blood platelets do not constitute a normal element of this fluid. The delayed clotting of lymph depends rather upon a deficiency in the contained thromboplastin, and consequently upon a relative excess of antithrombin. Such evidence suggests the possibility that some tissue in addition to the bone marrow is concerned with prothrombin formation. This view is supported also by the knowledge, to which reference has already been made, that the elaboration of fibrinogen depends upon the combined activity of at least two organs,—liver and intestine. The maintenance of the prothrombin equilibrium of the blood is obviously so important for the organism that the assumption that other tissues or organs participate in this process harmonizes better with our present conception of the factors of safety in physiological processes emphasized by Meltzer.

Our experiments afford no support for or against the view of Nolf and others that liver cell activity is essential for prothrombin production, nor do they make it clear what tissues besides the marrow play a part in its elaboration.

#### CONCLUSIONS.

1. Subcutaneous injections of benzol in rabbits produce marked destructive changes in the hematopoietic organs, especially in the myeloid tissue.

2. Benzol poisoning registers a change not only in the formed elements of the blood, but also in the factors of coagulation.

3. The circulating prothrombin is considerably reduced in amount and in most instances animals in which such a diminution occurs show aplasia of the bone marrow.

4. The association of extreme aplasia of the marrow without a fatal diminution in the circulating prothrombin suggests one of two possibilities: either other tissues and organs in addition to the bone marrow are concerned with prothrombin formation; or a minimum amount of myeloid tissue suffices to maintain the quantity of prothrombin above a dangerous level.

5. The myeloid tissue plays no part in the production of anti-thrombin.

6. Bone marrow activity is not essential for the production of fibrinogen.

In conclusion we desire to express our appreciation to Dr. Katherine R. Drinker for numerous fibrinogen determinations, and to the Pathological Department of the Peter Bent Brigham Hospital for many courtesies.

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# THE HISTOGENESIS OF CHRONIC URANIUM NEPHRITIS WITH ESPECIAL REFERENCE TO EPITHELIAL REGENERATION.\*

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PLATES 23 TO 30.

## INTRODUCTION.

Since the appearance of Dickson's (1) original work on the production of chronic nephritis by the administration of uranium nitrate, comparatively little morphological investigation has been done on this subject. With the exception of Christian (2) and Suzuki (3), investigators have turned their attention to the functional study of uranium nephritis, especially in its acute stages, and to the related condition of edema formation. Among those who have studied the former subject may be noted Folin (4), MacNider (5), and Christian (6); while the latter question has interested Richter (7), Schlayer and Hedinger (8), Georgopoulos (9), and, in this country, Pearce (10). The results of these studies have cast much light not only upon the effect of the renal lesion on the function of the kidney, but have also suggested a possible explanation for the progression of acute uranium lesions into chronic processes.

It has long been known that there is little or no tendency towards the production of a progressive chronic nephritis after the acute degenerative lesions following most of the renal poisons, whereas the development of such a progressive chronic nephritis is constant after acute lesions produced by uranium. Ophüls (11) has shown that there is no such tendency to the formation of a chronic nephropathy after acute chromium nephritis, a fact which is generally admitted by those who have studied the action of the different toxic agents (Suzuki (3)). It is true that slight interstitial lesions have been described by some writers, following the administration of chromium salts (Kabierske (12), Pander (13), and Smith (14)), but there is never the marked connective tissue proliferation with subsequent shrinkage which is so regularly seen in uranium poisoning.

The hypothesis advanced by Dickson to explain this fact held that there was a stimulative irritation of the connective tissue elements coincident with the destruction of the epithelium. This view was supported by the progressive nature of the connective tissue proliferation, its development around the blood vessels,

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and by the previously mentioned functional work which showed uranium to be a vascular as well as an epithelial poison.

Ophüls (15) in discussing the relation of acute epithelial degenerations to connective tissue proliferation says:

If any irritant acts simultaneously on both the epithelium and the connective tissue this (*i. e.*, early epithelial degeneration and latter connective tissue proliferation) must necessarily happen. The epithelial changes are early because the degeneration can and does occur within a short time, whereas the more slowly developing changes in the connective tissue show much later, necessarily. . . . Moreover, it is plainly brought out by all experimental evidence on this question that the more chronic the process the less marked the lesions in the epithelium and the more pronounced the interstitial changes. . . . I believe, therefore, that, on the contrary, the experimental evidence furnishes almost positive proof that the changes in the epithelium and the connective tissue are coördinate, not subordinate, to one another. They are the effect of the same cause acting simultaneously on different tissues, with different vulnerability and different reaction time, so far as visible changes are concerned.<sup>1</sup>

The opposite view of the relation of the two processes has been taken by Aschoff (16) and his pupil Suzuki. They made use of vital staining in an extensive study of the morphology of the renal secretion under normal and abnormal conditions, such as in the acute nephritides following different renal poisons, and showed that when uranium is injected in moderate doses it affects mainly the terminal part of the proximal convoluted tubule ("*Hauptstück*"), and so differs in its action from chromium, corrosive sublimate, arsenic, and cantharidin, which affect other parts of the same segment of the renal tubule. In chronic uranium nephritis, which they found regularly after the acute lesion, the connective tissue proliferation starts in that region where the epithelial damage was greatest, *i. e.*, in the outer stripe of the outer zone of the medulla (Peter (17)), for there the terminal divisions of the proximal convoluted tubules lie. They compare this process with the connective tissue proliferation observed in hydronephrotic atrophy of the kidney. This analogy they base on the assumption that regeneration of the renal epithelium proceeds from the upper part of the descending loop of Henle, which part is destroyed by the action of the uranium, and that the proliferation of the epithelial cells is hindered by the necrotic masses which fill the tubule at this point. They believe that there is a consequent collapse of the tubule in this region, with resulting atrophy of inactivity of the tubule above, and that the connective tissue, though primarily unaffected, proliferates and fills in the dead space.

These contentions of Aschoff and Suzuki all hinge on the exact mode of regeneration of the epithelium, as is evident from the fact that Suzuki's hypothesis is based upon the assumption that regeneration occurs chiefly in the upper portion of the descending limb of Henle; and still a review of the literature shows that remarkably little investigation has been done on this subject either before their work, or as a consequence of it. Beyond the casual statement that regeneration occurs after an acute nephritis, either spontaneous or experimentally produced, little detail is given of the process. Experimental investigations have

<sup>1</sup> Ophüls, W., *loc. cit.* (15), p. 489.

been largely confined to regeneration following mechanical insults, such as puncture, cauterization, ligation of the renal artery, or freezing the cortex. Podwyszożki (18) seems to have been the pioneer in this work, and the studies of Ribbert (19) and Hochhaus (20) have also dealt with the reparative processes in kidneys so treated. It is at once evident, however, that the results of such a gross attack on the renal elements can be applied only to a limited extent to the regeneration following the production of lesions by toxic agents which reach the kidney through the blood stream.

The only extensive study of regeneration following the administration of renal poisons has been made by Thorel (21), although Cornil and Toupet (22) have given a brief description of the mitoses which they observed in the late stages of cantharidin nephritis. Thorel made a systematic study of the reparative changes which occurred during the first nine days of chromium poisoning, and the summary of his results will be discussed during the review of the findings in the experiments which we have performed.

The descriptions of regeneration in human nephritides are of interest, especially in those cases where the newly formed cells were not of normal morphology, as it will be seen that similar conditions are found in the regenerated epithelial cells following uranium nephritis. Thorel (23) has noted atypical mitoses, hypo- and hyperchromatic figures, and the resulting production of a markedly atypical epithelium in a case of secondary contracted kidney; and Rösle (24), Tilp (25), and Oertel (26) have described the formation of giant-cell-like complexes in chronic human nephritis. The work of Heineke (27) should also be mentioned, as it gives a remarkable series of cases of corrosive sublimate poisoning in man, ranging from those in whom death occurred in a few hours, to those who survived for several weeks.

A new method of investigation of experimental nephritis has been afforded by the recent advances in vital staining. While no attempt will be made to review the general literature of this extensive subject, a brief resumé of its application to the study of renal morphology, both normal and pathological, will be given. A more detailed discussion is contained in the monograph of Policard (28).

The earlier studies on the excretion of dyes by the kidney, especially of carmine, were made by Chrzonszczewsky (29) and Ribbert (30). In these investigations, as well as in the articles of the writers who followed them, the carmine was described as being excreted in the convoluted tubules, proximal and distal, and in the ascending loop of Henle. This was the accepted interpretation until the work of Suzuki (3) showed that excretion, or, better, "*Speicherung*," was confined to the proximal convoluted tubule, and did not occur to any extent in any other part of the renal tubule. Suzuki also showed that the proximal convoluted tubule could be divided by means of the amount of vitally stained granules which its different divisions contain, into a proximal division adjacent to the glomerulus where the granules are numerous and arranged in the form of rods; a middle division in which the granules are much fewer in number, but are still arranged in rod-like formations; and lastly a terminal division in which there are but few granules scattered irregularly throughout the renal cells. This last division corresponds roughly with the "*Spiralrohr*" of earlier writers, and can be further differentiated from the ascending loop of Henle whose diameter and epithelium are very similar, by the possession of a cuticular seam, the "*Bürstenbesatz*."

Peter's work (17) has shown that the "*Spiralröhre*" ("*partie terminale*" of Policard) lie in the same region of the kidney as the ascending loops of Henle, i. e., in the outer stripe of the outer zone of the medulla, and that owing to the similarity of their epithelium and the difficulty of properly preserving the delicate cuticular seam, these two divisions of the renal tubule are easily confounded.

Suzuki's application of this method to the various experimental nephritides revealed the fact that the different toxic agents have a selective action on the different parts of the proximal convoluted tubule. For instance, chromium salts affect the heavily granulated division I, uranium affects the terminal sparsely granulated division III, and to a slight extent the moderately granulated division II, while sublimate and cantharidin affect almost exclusively the terminal division III. More complete details of the action of these toxic agents are described in Suzuki's monograph.

A further aid which the method of vital staining lends to the study of experimental nephritis is the behavior of the newly regenerated epithelial cells to the vital stain. It was first pointed out by Ribbert (31) that the newly regenerated cells do not contain the deposits of carmine which are seen in the original renal cells, and that by the application of vital staining a comparatively easy and certain method is obtained for distinguishing these two classes of cells. As will be seen in the discussion of Thorel's study of regeneration following chromium nephritis, this is often a matter of considerable difficulty with the ordinary methods of histological examination.

In the present study an attempt has been made to follow the development of the nephritis from the earliest acute stage through the successive changes which lead to a marked chronic nephritis. Not only have the changes in the regenerated epithelial elements been studied, but especial attention has been given to the early development of the connective tissue and to the origin of this chronic fibrosis. In addition, the interpretations of Suzuki, especially in so far as they deal with the method of production of the chronic lesions in uranium nephritis, have been the subject of careful analysis.

#### METHODS.

A combination of the methods of Thorel and Suzuki was used. An attempt was made to produce an acute nephritis of fairly constant severity in a vitally stained animal and to observe the progress of the condition by sacrificing the animals on successive days. Guinea pigs were used for the majority of the experiments for two reasons. First, as Thorel has pointed out, it is necessary to produce the greatest possible damage to the kidney which is compatible with life in order to obtain constant results in the severity of the acute nephritis

in the various animals and in the consequent progress of the condition. Rabbits are much more susceptible to the poisonous action of renal irritants and a large percentage succumb during the acute stage of the intoxication. Moreover, they are much more susceptible to spontaneous chronic nephritis than are guinea pigs (Ophüls (32)), and its possible occurrence is apt to lead to confusion with the experimentally produced lesions. Even with these precautions there is always the individual susceptibility of the animal to be considered, and this can only be avoided by a large series of experiments. In some cases where it was necessary to prolong the life of the animal for two weeks or more, a smaller dose of uranium was given, and in these cases, though the animal apparently recovered from the initial acute attack, it eventually died of the resulting chronic nephritis (compare guinea pig 51, figure 1). In detail the procedure was as follows. The guinea pig received five milligrams of uranium nitrate in a 1 per cent. solution, and on the two following days, two cubic centimeters of a 5 per cent. solution of carmine in saturated lithium carbonate solution. The animals were killed at the proper time, unless death resulted spontaneously, and the kidneys were fixed immediately in 10 per cent. formalin. A brief washing in running water, followed by imbedding in paraffin section, and a light counterstain with hematoxylin, and Van Gieson solution completed the technique. In the later stages, after the tenth day, the animals were sacrificed every other day.

For the purpose of comparison a small series of rabbits was used, which also received five milligrams of uranium but no carmine, and a series of white rats which received uranium, chromium, or sublimate, with subsequent vital staining.

The number of animals used was: guinea pigs receiving uranium, fifty-four; white rats, eighteen, six receiving uranium, chromium, and sublimate, respectively; and twenty-two rabbits which received uranium alone. In order to save space and to avoid repetition, the individual protocols of the animals have been omitted, and only a general description of the process observed in them is given.

## THE APPEARANCE OF THE NORMAL KIDNEY IN VITAL STAINING.

But brief mention of our findings in the normal kidney as studied by vital staining will be given, as our results coincide perfectly with those of Suzuki (3). The proximal convoluted tubule alone shows the deposit of carmine granules, and in this region the number of granules decreases as one departs from the glomerulus. In the following descriptions we shall therefore use Suzuki's terminology and refer to the different divisions as divisions I, II, and III, numbering from above downwards, the heavily granuled division with rod formation, the medial lesser granuled division, and the terminal, sparsely granuled division.

The distribution of the vitally stained granules may be followed in serial sections, but a more certain method is that of maceration in hydrochloric acid with subsequent teasing out of the kidney tubules. In kidneys heavily stained with trypan blue the granules will resist the action of the acid if not left too long, and appear a dark green color. Figure 12 shows the glomerulus and the proximal convoluted tubule (*Hauptstück*) with a decreasing number of stained granules, as one approaches the narrow descending limb of the loop of Henle. Figure 13 shows the distal convoluted tubule (A) (*Schaltstück*) connecting with the collecting tubule (B). Though the cells of the former are somewhat more granular than the transparent cells of the collecting tubule, no vitally stained granules are present in them. Similarly no granules are found in either limb of the loop of Henle or in the collecting tubules.

## ACUTE NEPHRITIS FOLLOWING VARIOUS TOXIC AGENTS.

(FIRST TO THIRD DAY.)

## URANIUM.

*Guinea Pigs.*—Immediately following a moderate dose of uranium nitrate, five milligrams for a large guinea pig, the lesions observed are striking in constancy and localization. Division I and, as a rule, division II of the proximal convoluted tubule are intact, and the epithelium shows the normal deposit of vitally staining granules. As one approaches division III, the damage to the renal cells becomes evident. The cytoplasm of the renal epithelium is destroyed

and the lumen of the tubule is filled with a granular detritus which is stained a diffuse pink by the carmine. The nuclei are either pycnotic and stained a bright red, or show various stages of disintegration. A few cells persist in which the nuclei are still normal in appearance and in which the cytoplasm shows but a slight amount of change as indicated by irregularities in the arrangement of the vitally staining granules. This persistence of the cells in the degenerated areas must be kept in mind, as it is a point of importance in the later changes in the epithelial structures. The ascending loops of Henle show a slight amount of damage in the more severe cases, perhaps somewhat more commonly in our specimens than Suzuki has described.

It will be seen that the greater part of the damaged tubules lie in the outer stripe of the outer zone of the medulla, a fact which can be recognized in the gross appearance of the kidneys. In rabbits in which the various divisions of the medulla are well marked, this distribution is easily seen as a broad band of deeply vitally stained, opaque degenerated tissue extending between the cortex and the inner stripe of the outer zone of the medulla.

The other divisions of the kidney are practically normal. In the collecting tubules are a varying number of hyaline casts, which, if the injection of carmine has but shortly preceded the death of the animal, are stained with carmine. Very few casts, exclusive of the masses of epithelial detritus, are seen in the proximal convoluted tubules or the loops of Henle.

To sum up, the process is limited to division III of the proximal convoluted tubule, but as the severity of the lesion increases, the damage ascends towards division II, and is also seen in the ascending limb of Henle's loop. A fact of much importance is the persistence of a few cells with normal nuclei and only slightly disturbed cytoplasm. When one considers the small part of the tubule seen in one section, the number of these persisting cells must be considerable.

*Rabbits.*—The lesion in the rabbit kidney is similar to that observed in guinea pigs except in two respects. The kidney of the rabbit is relatively much more susceptible to all irritants than is that of the guinea pig. The lesions are therefore much more extensive, and with the same dose as that given to the guinea pigs, five milli-

grams, there is necrosis of practically the entire proximal convoluted tubule, as well as marked degeneration in the ascending loop of Henle. These tubules are filled with dense masses of granular detritus, and many casts are seen in the collecting tubules.

Another striking feature is the change seen in the glomeruli of many of the animals. Christian (33) has described various lesions in the glomeruli of uranium rabbits, hyaline droplets in the capillary walls, fibrin thrombi in the capillaries, dilatation of the capsular space with granular material, and proliferative lesions affecting the capillary endothelium. In a comparatively large number of our rabbits, lesions were observed in the glomeruli, consisting as a rule of a filling of the capsular space with granular material, and pycnosis of the endothelial nuclei in the loops.

*White Rats.*—(Figure 2.) The lesions observed in white rats differed in no way from those observed in guinea pigs. The tendency to repair is great in these animals, and the regenerative changes, though essentially the same, appear more quickly than in guinea pigs and are therefore present before the end of the third day.

#### CHROMIUM.

(Figure 3.) To confirm Suzuki's description of the marked difference in the action of chromium and uranium on the renal tubule, a few white rats were injected with two milligrams of potassium bichromate followed by carmine.

The difference from the histological picture of acute uranium nephritis is evident at first glance. The heavily stained divisions I are lacking, and their place is occupied by tubules which consist of the bare membrana propria which are filled with a pinkish granular detritus. As one approaches the medulla a few normal vitally stained tubules are seen, and in the outer stripe of the outer zone of the medulla are seen the faintly vitally stained divisions III, practically in normal condition. In severe cases pycnotic nuclei are present even in this last division, but never the severe necrosis observed in uranium poisoning. The glomeruli are normal in all cases.



## SUBLIMATE.

The acute sublimate nephritis resembles more nearly that observed after uranium administration. The heavily stained division I is intact, and in all but the most severe cases division II is also. Division III shows a marked necrosis of the epithelium, which extends well down into the medulla. This extension downwards is to a lower level than is ordinarily seen in uranium nephritis, but it is difficult to say whether this is due to the size of the dose employed or to an intrinsic difference in the action of the two toxic agents.

THE REGENERATIVE CHANGES IN THE EPITHELIUM FOLLOWING  
ACUTE URANIUM NEPHRITIS.

The picture of acute uranium nephritis described above holds for the first three days of the process. It is only at the end of the third day, and in guinea pigs often during the fourth day, that the process of regeneration becomes at all marked. In such preparations one is at once struck by the appearance of the affected tubules. The greater number still contain granular detritus which more or less completely fills the lumen. In many cases the tunica propria is still bare, but in others two or three comparatively enormous nuclei are seen which are covered with a rather indistinct layer of protoplasm. These nuclei, as a rule oval in shape, possess a relatively large amount of deeply staining chromatin, which is arranged in a loose thread-like network, and possess one, and at times two, large nucleoli. The long axis of these nuclei is indifferently arranged in relation to the axis of the tubule, a fact which becomes quite striking in the later stages when a large number of them is present.

In the same tubules in addition to the large nuclei are smaller ones, approximating the size of the normal renal cell or even smaller. Many of these show no detail in their chromatin, but present a dense, and when stained with hematoxylin, a black appearance. In preparations where the vital staining has been especially intense, the cytoplasm shows a few scattered carmine granules. There can be no doubt that in these we have to do with the original renal cells whose nuclei have been severely affected by the uranium, but which still retain a certain amount of vitality. They correspond to the cells which Thorel has described in various degenerative processes and which he believes are evidences of early regeneration.

In the neighborhood of these large newly formed nuclei one can often find mitotic figures in varying stages of division. The number of mitoses is always small as compared with the number of the regenerated cells, a fact which has been emphasized by many writers. The spindle figures seen are entirely normal, there being no evidences of asymmetrical distribution of the chromatin (Thorel (23)).

Besides these spindle figures, we would call especial attention to the premitotic and early stages of the process of mitosis. These have not been described in detail by other writers, and if one considers their number in addition to the spindle forms, the discrepancy between the number of mitotic figures and the active nature of the regenerative process is much reduced. The preliminary changes which the nucleus commonly undergoes prior to the process of indirect division are all well shown in the regenerating tubules. One of the commonest observations is the enlargement of the nucleus, with the formation of a loose spirem. In other nuclei where the process is more advanced, the nuclear membrane is dissolved and the chromatin lies free in the cytoplasm ready for the formation of the aster. In none of these early karyokinetic figures can any abnormalities be found.

It is possible that some of the large nuclei described previously are not newly formed cells, but are the original renal cells which have withstood the attack of the toxic agent and have enlarged prior to division. This would, however, be a rather finely drawn distinction, as the large nuclei must either be cells which have but recently undergone division, or those which are about to divide.

The protoplasm surrounding these new large nuclei never shows any evidence of the carmine granules. This accords with the descriptions of Policard and of Ribbert, who both called attention to the absence of vital staining in dividing and recently divided renal cells. The contrast with those original cells which have survived the action of the toxic agent and which contain carmine granules is marked, though the scarcity of carmine in the cells of division III necessitates the study of only those specimens which are intensely stained to demonstrate this fact.

As time passes these regenerative changes become more and more

pronounced. At the end of the fifth or sixth day the large nuclei have so increased in number as to alter materially the structure of the tubule at this point. There has resulted an excessive formation of these new elements so that they now lie in masses, with little evidence of division in the surrounding cytoplasm. In this way syncytium-like masses are formed which may apparently close the lumen of the tubule (Figure 4 A). We must, however, remember the relatively small part of the tubule which is seen in one section, before drawing absolute conclusions as to the permeability of the tubule, for, as will be seen later, the permeability of the tubule is an important factor in the hydronephrotic atrophy theory.

Another appearance which is fairly common is the formation of giant-cell complexes, consisting of from six to twelve nuclei in a mass of cytoplasm which projects with a rounded contour into the lumen of the tubule (figures 4 B and 5). Their significance is the same as that of the syncytium-like masses described above, but their peculiar shape and the fact that they are described under the name of giant-cells in human chronic nephritides warrants a separate consideration. These are especially common in tubules where regeneration is most active, and for the same reason are especially common in rat kidneys. When we consider the isolation of the surviving renal cells as described in the first days of the nephritis, it seems most likely that these nuclei are the offspring of the nucleus of such an isolated renal cell, which have become heaped up as a result of the rapid proliferation.

The covering of the *membrana propria* proceeds slowly from the large multinuclear masses. One can see the protoplasm with the nuclei enclosed creeping along the supporting membrane (figure 5), and by this method the tubule is gradually covered with a layer of large cells which differ widely from the original epithelium. Their nuclei are still large and the chromatin stains deeply. The cytoplasm as well stains more deeply with ordinary stains than that of the normal renal cell, so that the tubules resemble superficially the collecting rather than the convoluted tubules. Another striking appearance is the excessive production of cellular elements. In many of the tubules with regeneration the epithelium consists no longer of one layer, but of irregularly, densely packed cells with

nuclei. These large oval nuclei are either placed in palisade-like formations (Thorel) or with no orderly arrangement, their long axes lying in all directions.

The normal division I of the proximal convoluted tubule with its heavy granulations of carmine undergoes relatively little change throughout the process of repair. An occasional mitotic figure is seen in it, or a group of enlarged nuclei, but there is little evidence of cellular proliferation as compared with the degenerated region of the tubule. Similarly the narrow descending limb of Henle's loop shows even less proliferation in its epithelium. The significance of these facts will be discussed in the critical review.

The effect of the detritus and casts on the regenerating cells may well be considered at this point. In the earlier stages we have seen that division III of the proximal convoluted tubules is almost entirely filled with a rather loose granular detritus, whereas the well formed hyaline casts are almost entirely limited to the collecting tubules. This condition persists throughout the first three days, and as there is little or no regeneration during this period, it is more than likely that the proliferation is at least partly retarded by the inhibitive effect of the pressure from the detritus. When regeneration does begin, however, it overcomes this resistance quite readily. The protoplasm containing the large nuclei creeps along the membrana propria beneath the detritus, and though the pressure of the latter flattens the new cells somewhat, the tubule is ultimately covered with epithelium. The shrinkage and condensation of the granular material removes much of the restraining influence and the flushing of the tubule carries the detritus into the lower levels of the kidney. As we shall see later, in considering the changes in the connective tissue, this is the most favorable outcome, for in many cases another process which leads to permanent damage to the kidney results.

Phagocytosis of the necrotic material by the regenerating epithelial cells as described by Heineke (27) has not been observed in the kidneys of any of the animals, irrespective of the toxic agent used.

The further development of the giant-cells and syncytial masses consists chiefly in an equal distribution by active growth extension of the irregularly arranged nuclei over the membrana propria (fig-

ure 5). By this means the prominence of the giant-cells is decreased and they are ultimately levelled to a uniform height, so that a single layer of cells now covers the membrana propria. In many cases, however, there is still an excess of nuclei, and these may form an irregular second layer of cells around the lumen of the tubule. Although the nuclei decrease somewhat in size and lose their vesicular appearance, they are still larger and more deeply staining than those of the normal renal cells and their protoplasm still stains deeply.

The assumption of a normal morphology by these regenerated cells in uranium nephritis is doubtless greatly hindered by the connective tissue proliferation which develops conjointly with the epithelial changes. By the tenth day, in those tubules in which regeneration occurs, there is as a rule no sign of degenerated, *i. e.*, necrotic cells, nor are there spaces of any extent yet to be covered with epithelium, yet as late as the 117th day the regenerated epithelium which is now surrounded by connective tissue still differs widely from the normal renal cells. It is, therefore, improbable that the regenerated epithelial cell in uranium nephritis ever regains the morphology of the original renal cell. As there is no sign of renal cells of abnormal morphology outside of these areas of connective tissue proliferation, the possibility cannot be denied that some of these apparently intact original cells are regenerated cells which have become indistinguishable from the normal cells around them. If one follows the process through the succeeding days, the unlikelihood of such an occurrence is evident, as the primary degeneration is not diffusely distributed, but confined to definite localized areas which correspond to the later appearing patches of connective tissue.

#### THE PROLIFERATIVE CHANGES IN THE CONNECTIVE TISSUE.

In the first few days of the acute nephritis the membrana propria which has been denuded of its epithelial covering remains apparently passive. It is not until the sixth or seventh day, or in severe cases, as late as the ninth, that one notices any reaction on its part.

At this time the cells of the membrana propria, which are normally few in number, small and thin, and enclosed in the fibrils of connective tissue which compose the membrane, begin to show signs

of proliferation. Instead of isolated cells they appear in groups of two or three, are twice their normal size and so project somewhat beyond the limits of the fibrous membrane into the lumen of the tubule (figure 6). The connective tissue fibrils are often separated by this process, the normally homogenous appearing membrane showing its constituent elements. These appearances are best seen in those tubules in which there is little or no regeneration, but which are filled with granular detritus.

At about the same period there occurs in such tubules a marked infiltration of the detritus with leucocytes, principally polymorphonuclears and some lymphocytes, which fill the contents of the tubule to such a degree as almost to replace the formerly light staining detritus (figures 6 and 7). At the same time in the intertubular spaces connective tissue cells may be seen in active proliferation, as is evidenced by their increase in number, large size, and fusiform shape. These cells have the typical morphology of the fibroblast, with elongated nuclei and scanty protoplasm.

Not only do these proliferating fibroblasts fill in between the tubules, but invasion of the leucocyte-infiltrated detritus occurs. The typical fusiform nuclei are seen scattered among the leucocytes. Beside fusiform nuclei, many nuclei of bizarre shape, some not unlike amitotic figures, are common, and in the later stages connective tissue fibrils are formed (figure 8).

It is evident that this growth on the part of the connective tissue, which as we have stated becomes evident on about the ninth day, can lead not only to an obliteration of the lumen by ingrowth, but to a collapse of its walls as well. The polymorphonuclear phagocytes remove the granular detritus, leaving the field open to the lymphocytes and to the fibroblasts which rapidly grow in and form connective tissue fibrils.

The effect of the obliteration of division III of the proximal convoluted tubule makes itself felt especially on that part of the renal tubule lying proximal to the occlusion. As these distension phenomena have received much attention by Aschoff and Suzuki in their theory of the pathogenesis of the uranium contracted kidney, we shall return to the early stages of the nephritis for a complete consideration of them.

In the acute stages of the nephritis, when the entire division III of the proximal convoluted tubule is packed with a dense mass of granular detritus and when the number of casts is at its maximum, there is no dilatation of the proximal convoluted tubule, nor do the capsules of the glomeruli show any distension (figure 2). This fact is easily determined in vitally stained kidneys, as here the heavily granulated tubules would be concerned. In many specimens, however, there is considerable distension of the collecting tubules, both in the medulla, medullary rays, and in the cortex, and to some extent the same appearance is seen in the distal convoluted tubule and the ascending limb of Henle's loop. The dilatation of these latter divisions may well be due to the hyaline casts in the lower levels of the collecting tubules in the medulla, as the amount of distension varies directly with the number of these casts.

A factor which must be considered in relation to the dilatation of the tubules is the variance observed in the lumen of the tubule in different functional states of the renal secretion. It is well known that there is a comparative distension of the glomeruli and the tubules throughout the kidney in active diuresis. This source of error is easily avoided, however, by giving no carmine injection immediately before the death of the animal, thus avoiding the period of renal hyperexcretion which immediately follows the injection of the vital stain.

As the process of connective tissue development proceeds, a moderate degree of distension appears, which gradually increases with the contraction of the fibrous tissue and the resulting tubular destruction. The latter, as we have seen, begins about the ninth day, and similarly at about this period beginning distension of the upper portion of the proximal convoluted tubule becomes evident. By the twenty-first day there is a marked dilatation of Bowman's capsule, with the formation of glomerular cysts, and some dilatation of the heavily granulated proximal convoluted tubules, though these last divisions never show any extreme distension (figure 9). There is, therefore, little comparable to the process of connective tissue formation that we find in hydronephrotic atrophy, as the distension in this case is an effect and not a cause of connective tissue formation.

The processes described in the epithelial regeneration and in the

connective tissue proliferation result in a striking change in the morphology of the kidney cortex. Throughout its lower layers one sees areas which stand out distinctly from the normal tubules and which give a mottled appearance to the section. In the earlier stages, when the connective tissue is just beginning to proliferate and the necrotic material is infiltrated with leucocytes, these areas are in the form of ill defined, rather widely scattered strands of deeply staining nuclei (figure 10). As the growth of connective tissue increases, these diffuse strands coalesce to form denser areas of mixed proliferating connective tissue and enlarged regenerated epithelial cells, which are sharply demarcated from the lighter staining normal tubules (figure 1). These areas persist throughout the succeeding days, until the beginning of the shrinkage of the connective tissue and the collapse of the affected tubules (fifteenth to twentieth day). By these two processes the areas are contracted to much less than their former size and by extension of the connective tissue growth around the upper divisions of the proximal convoluted tubule, glomeruli are finally included within the fibrotic patches (figure 9).

By means of this extension around the primarily unaffected proximal convoluted tubules, these areas, originally located deep in the substance of the cortex, gradually reach the cortex corticis, and by means of the connective tissue shrinkage which is progressing in them form the scars or dimples seen on the surface. Originally broad laterally, these areas now form narrow bands extending from the junction of the medulla and cortex to the capsule in ray-like processes, which become more fibrotic as time elapses.

The relation of the connective tissue proliferation to the blood vessels is a difficult one to decide. As practically all the later specimens show, the areas of connective tissue proliferation lie in proximity to the large vessels. This fact, originally pointed out by Dickson, has been explained by the opponents of the inflammatory theory as being a coincidence, as the vessels are most numerous where the epithelial damage is most marked; *i. e.*, at the junction of the cortex and medulla. This cannot be denied, nor is there any evident way to settle the question morphologically. A more detailed discussion of this problem will be given in the critical review.



## CRITICAL REVIEW.

In comparing the results of the present investigations with previous work on allied subjects, we shall first consider the regeneration of the epithelial elements.

The experiments of Thorel (21) are the only ones which correspond closely with the methods employed in our work. In the regeneration following bichromate poisoning in rabbits, he describes the sudden appearance on the third day of peculiar, deeply staining nuclei in those tubules which have been cleared of detritus. Mitotic figures are common, five or six to the field, and of varying size. During the fourth and fifth day there is an extension of this process of the clearing away of detritus and the springing up of small cells. In the late period (fifth to ninth day), there is a break in the histological picture of the process due to the sudden appearance of large nuclei. These large cells are of various shape, oval, round, cuboid, or irregular, due to the pressure exerted upon them by the neighboring cells. In some places they possess separate protoplasm, while in other regions there is a syncytial formation. The nuclei measure 8 to  $10\mu$  as contrasted with  $6\mu$  of the normal renal nuclei, and are of an oval shape with a vesicular structure, a distinctly formed chromatin network, and one or two nucleoli.

In speaking of this sudden change and its relation to the preceding process, Thorel says: In our investigations the further course of the process of healing *unfortunately* breaks off so sharply and suddenly on the fifth day of the nephritis *that it was impossible to trace the blending of the above described processes and the transition of the individual newly lined tubules with their histologically so varying cell-lining into the harmonious regenerative processes after the fifth day of the nephritis.*<sup>2</sup>

The marked differences in the above description and in the processes described here for uranium poisoning are easily reconciled if we consider the small nucleated cells not as regenerated cells, as Thorel did, but as the original epithelium which has been slightly affected by the toxic agent and not entirely destroyed. In the first two days of the uranium nephritis many cells according perfectly with the small nuclei of Thorel are seen scattered throughout the damaged regions of the cortex. Not only do they closely resemble pycnotic nuclei, but, as we have shown, their reaction to the vital stain is not that of regenerated cells, but of the original epithelium. We would, therefore, class these cells not as newly regenerated cells, but as partially affected persisting renal cells. Such a conclusion simplifies the understanding of the "late stage" of Thorel very much. The large vesicular nuclei described as appearing on the fourth or fifth days are the beginning of the regeneration, not a sudden appearance of cells which have no relation to the preceding processes, a phenomenon which Thorel could not explain. The appearance of these large nuclei in bichromate nephritis and their similarity to those found in uranium nephritis are shown in figure 11.

Other writers, Arnold (34) in tuberculosis of the kidney, and Friedländer (35) in scarlet fever, have described apparently similar cells as newly formed

<sup>2</sup> Thorel, C., *loc. cit.* (21), p. 407. The italics are in the original.

elements, and Tilp (25) finds them commonly in his series of human renal affections. The latter is, however, somewhat doubtful of their significance, as he says: I had rather the impression that many of these distorted and deeply staining cell nuclei, as Thorel describes them, must not be altogether regarded as newly formed, but that we might have to do with nuclear degeneration besides newly formed cells.<sup>3</sup>

Giant-cell complexes, which have been described by many authors (Tilp, Rössle, Oertel), are commonly found in uranium nephritis, especially in rats, where the regenerative process is very pronounced. Their number seems to be directly proportional to the rapidity of the regenerative process, and this rapidity of growth is the explanation of their origin. A solitary cell, or group of cells, divides and the resulting daughter cells redivide with such rapidity that there is a heaping up of nuclei at a localized point. As we have seen, these giant-cells ultimately spread out over the membrana propria and subdivide and so disappear. A similar fate has been described for them by Tilp. The theory of Oertel that they represent an inflammatory hyperplasia receives little support from the present experiments. It is interesting to note that somewhat similar appearances are described in active regenerative changes in the liver. The knobs described by Klebs, Meder (36), and others are probably expressions of the same process that we see in the giant-cell formation in acute nephritis.

Mitotic figures are common and would seem to be the predominant method of cell division in the regenerating epithelium. Many authors have mentioned their scarcity in proportion to the great number of regenerated cells that are seen. A careful examination in such cases will show a large number of early mitotic figures, such as the early prophase with spirem formation. From the descriptions and figures of most writers, the late prophase and anaphase with spindle formation and diaster have received the most attention, while no mention is made of the early stages of the process. By the inclusion of these frequent prophases, their number more nearly approaches that expected with the rapid regenerative changes.

In none of the mitotic figures seen was there any evidence of abnormality, such as pluripolar spindles, hyper- or hypochromatic figures, or unequal distribution of the chromatin, as is described by Thorel (23). The part played by amitosis in the regenerative processes is difficult to decide. It may have significance in the rapid division resulting in giant-cell formation, as suggestive nuclear forms have been observed here, with a corresponding absence of indirect figures. Tilp makes the same suggestion, but has seen spindle formation in the giant-cells.

The seat of origin of the new cells is a point which must be considered, as Aschoff makes use of this factor in his theory of the pathogenesis of chronic uranium nephritis. In the observations of his pupil, Suzuki, the new epithelium covering the destroyed division III of the proximal convoluted tubule is described as arising primarily in the upper end of the narrow descending limb of Henle's loop, and to a lesser degree in the remnants of division III.

We have shown that even in the severe cases where the tunica propria is practically denuded there is a persistence of a few cells at some point whose nuclei

<sup>3</sup> Tilp, A., *loc. cit.*

are normal, although their cytoplasm, with its scanty carmine granules, may show some slight change. When one considers the small part of the tubule present in one section, it is evident that a considerable number of cells must escape the original injury and be capable of giving rise to new cells which will fill in the defect. Such was Thorel's assumption in chromium nephritis, though through the lack of a method of differentiation between original and newly formed cells, this fact then was not so easily accessible to direct observation.

In the narrow descending limb of Henle's loop we have failed to find any proliferation of practical importance. Some signs of cellular division are occasionally present, but the same can be said of the collecting tubules, where an occasional large nucleus or mitotic figure can be seen. In fact there is much more evidence of proliferation (mitotic figures, enlarged nuclei, and excess of nuclei) in the carmine-stained divisions I and II, which lie above the damaged area. Nor could the formation of the isolated giant-cells and syncytial masses be well understood if the newly formed cells which compose them had grown upwards from the descending loop of Henle. We therefore consider that the regeneration occurs directly in the surviving cells of division III of the proximal convoluted tubule.

Two methods by which gaps in the renal epithelium are filled have been described. Podwyszożki (18) laid most importance on what he termed "regeneration *per intussusceptionem*." An increase in renal cells at some distance from the gap, assisted by an individual cell hypertrophy, results in an increased pressure, by which the persisting cells which lie at the edge of the defect are pushed into and over the break in epithelium and so close it. Other writers, notably Ribbert (19) and Loeb (37), have described an active wandering of the epithelial cells into the defect, the former in the epithelium of the kidney and the latter in the epithelium of the skin of the guinea pig. Thorel, on the other hand, lays much weight on Podwyszożki's views and observes the development of areas of vicarious regeneration at a distance from the seat of damage.

In the present investigations the mechanism of the filling in of defects is almost entirely that described by Ribbert. The cells are seen fixed in various stages of active growth extension and their protoplasmic processes can be seen extending forwards, often beneath the mass of detritus which lies upon them. As we have previously stated, there is some evidence of proliferation in the intact, vitally stained divisions I and II of the proximal convoluted tubule. This might be considered analogous to the areas of vicarious regeneration described by Thorel, but the amount of such change is insignificant in comparison with the active processes in the damaged division III.

Before discussing the changes in the connective tissue, it will be well to review briefly the normal anatomy of the renal stroma. Disse (38) has given a review of the subject, and it is from his description, and the work of Rühle (39), that the following is taken. The connective tissue of the kidney, or stroma, is composed of a network of fibrils which Mall (40) has shown to be made up of reticular tissue and collagen fibrils. The fibrils arise directly from the adventitia of the larger vessels and surround the tubules, nerves, veins, and lymphatics. All the ramifications of the vessels are enclosed in these meshes, and, as Rühle has shown, the fibrils form the walls of the capillaries and smallest vessels, on which the endothelium lies directly. The border of fibrils which abuts the epithelium

of the tubules is somewhat condensed and forms the membrana propria. This structure has been studied by Rühle (39), who shows that the above mentioned fibrils of the stroma wind around the tubules in spirals, taking part in formation of the membrana propria, and then return, uniting with the perivascular connective tissue. Among the fibrils lie the spindle-shaped connective tissue cells, which in young animals send processes, which unite with, or constitute, the fibrils. The membrana propria, which appears in ordinary sections as a homogeneous membrane, is, therefore, only the condensed connective tissue of the kidney on which the epithelial cells lie. This, and the fact that connective tissue fibrils not only arise from the walls of the larger vessels, but even form the walls of smaller ones, are considerations whose importance in the pathological changes to be discussed cannot be overrated.

The importance of the vascular connective tissue stroma in the process of repair in the kidney has been recognized by many writers. Ziegler (41) states that an intracanalicular regeneration, as opposed to the regeneration of new tubules, is only possible when the vessel-bearing stroma is intact. If this latter is damaged there is either no regeneration, or only an abnormal epithelium is formed.

Aschoff (42) in speaking of reparative changes in the tubules says: If the tunica propria is destroyed under the influence of the inflammation, a rebuilding of the tubule in this location does not take place. Tilp (25) distinguishes two types of regenerative processes: that in which the vascular connective tissue apparatus ("*Blutgefäßbindegewebsapparat*") is not disturbed and in which there may be a complete return to normal morphology and function; and a type in which the stroma also is damaged and where repair is never complete. This latter type he describes in chronic Bright's disease, renal infarcts, tuberculosis, and tumors of the kidney.

We have seen from the description of the development of chronic uranium nephritis that there never results a morphological restitution of normal structure in the kidney, and that this lack of restitution is due to the development of connective tissue in the affected areas, which begins early and progresses steadily until the death of the animal. Two possibilities present themselves for the explanation of this fact. The connective tissue overgrowth may be due to a disturbance in the structure of the vascular connective tissue stroma, or it may be the result of a primary damage solely to the epithelial elements, with a passive ingrowth of connective tissue to fill in the dead space resulting from a collapse of the tubules. As we have stated in the introduction, the former is the view of Dickson, and is supported not only by his morphological studies, but also by the physiological investigations of those who have studied the function of uranium kidneys. A further morphological support to this theory has been given by the work of Baehr (43), who has shown that severe damage may be done the vascular apparatus, if the uranium reaches the kidney in high enough concentration. By injecting directly into the renal artery of rabbits he has produced necrosis and hemorrhage from the capillaries of the glomeruli. Chromium and other toxic substances did not produce any such condition, showing definitely that there is some specific action on the blood vessels in uranium poisoning.

The opposite view, of primary epithelial damage, has been upheld by Aschoff and Suzuki in a modified form, in which they liken the process to that observed

in hydronephrotic atrophy of the kidney. They claim that the insignificant regeneration in division III of the proximal convoluted tubule, due to the inhibitive effect of the necrotic masses and the destruction of the point from which regeneration occurs (upper part of the descending limb of Henle's loop), leads to a collapse of the tubule with resulting inactivity atrophy of the tubule proximal to this point, and resulting development of connective tissue around the atrophic tubule. They therefore characterize the kidney in chronic uranium nephritis as a "*tubuläre Schrumpfniere*," which has no homologue in the human nephritides, with the possible exceptions of pyelonephritic contracted kidneys, uric acid kidneys, and sclerotic foci proximal to renal cysts.

The hydronephrotic atrophy theory will first be considered. That part of the theory which has to do with the regenerative changes in the damaged tubule and the origin of the new cells has already been dealt with. A special study was made of appearances which might be interpreted as a hydronephrotic change, and as evidence of such a process the dilatation of the tubule above the point of obstruction was taken. It has been seen that dilatation of the proximal convoluted tubule and of the glomerular space is lacking until the twelfth day, a period considerably later than the beginning of connective tissue proliferation. In the earlier stages, although there is a marked filling of the tubule with detritus and casts, there is practically no distension. As the process proceeds and the connective tissue becomes more fully developed, the dilatation of the proximal convoluted tubule and glomerular capsule occurs and reaches its maximum about the twenty-fifth day, when marked connective tissue shrinkage is present. The collecting tubules show a dilatation from the first, due to the presence of many hyaline casts in the lower levels of the medulla, but there is never any evidence of connective tissue proliferation around them. To recapitulate, in the acute stages of epithelial degeneration, there is no sign of dilatation in the tubule above the seat of lesion, while in the later stages dilatation begins with the development of the connective tissue, and increases in direct proportion to it.

An especially good demonstration of the fact that the late dilatation phenomena in divisions I and II of the proximal convoluted tubules are due to connective tissue shrinkage is found in kidneys of animals suffering from a spontaneous chronic nephritis at the time of the acute attack from the uranium injection. In those areas where there is no spontaneous chronic process, division III of the proximal convoluted tubules shows the typical acute necrosis and its lumen is filled with detritus, yet there is no dilatation in the corresponding divisions I and II of the glomerular spaces above. In the areas of preëxisting chronic nephritis where there is marked proliferation of connective tissue and resulting collapse of the tubules, there is marked dilatation of the upper divisions of the proximal convoluted tubules and cyst formation in the glomerular spaces.

These facts cannot be reconciled with the hydronephrotic theory, but point directly to primary vascular connective tissue irritation with resulting proliferation. The same epithelial damage in division III of the proximal convoluted tubule is seen following the use of other toxic agents. In fact, in sublimate poisoning the epithelial degeneration is more severe than that which follows uranium administration, and the epithelial detritus is much more persistent and is often ultimately calcified, yet there is no tendency to the formation of a progressive chronic nephritis.

We therefore believe that a purely parenchymatous theory, one based solely on the reaction of the epithelial elements to the toxic agent, fails to explain the pathogenesis of the chronic uranium nephritis. The connective tissue reaction is too early and active to be considered a purely secondary filling in of dead space, nor can it be explained by a hydronephrotic theory. We therefore consider that the proliferation of the connective tissue in chronic uranium nephritis is due to a primary direct stimulation by the uranium, which so differs in this regard from the other renal toxic agents.

#### CONCLUSIONS.

1. Proliferation of connective tissue in chronic uranium nephritis is early and progressive and due to a direct stimulation on the part of the uranium.

2. The hydronephrotic theory fails to explain the phenomena observed in the progress of the chronic nephritis.

3. Carmine is deposited, as shown by Suzuki, in the proximal convoluted tubule only, and in different amounts in different levels of this segment of the tubule, so that subdivisions may be made.

4. Regeneration begins about the fourth day, but varies in proportion to the dose of renal irritant given.

5. The first cells to appear are large vesicular elements, and there is a gradual development of syncytium-like masses and giant-cells. These last are due to the rapidity of cell division.

6. Small, deeply staining nuclei, which are morphologically identical with the newly regenerated cells described by Thorel and others, are present from the first day of the acute nephritis and are persisting renal cells which have been slightly affected by the toxic agent.

In conclusion I wish to thank Dr. E. C. Dickson and Dr. W. Ophüls for their guidance and interest, and for their frequent aid in the progress of this work.

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## EXPLANATION OF PLATES.

## PLATE 23.

FIG. 1. Guinea pig 51. Uranium; killed on fifteenth day. The same region as the section in figure 10. The diffusely scattered strands of the earlier stage have coalesced to form a dense area of large regenerated epithelial cells and proliferated connective tissue which fills the lower half of the figure. A glomerulus



lies adjacent to this area and is being surrounded by the process, the effect of this being shown by the beginning dilatation of the capsular space. The divisions of the proximal convoluted tubules and collecting tubules at the upper margin of the figure are entirely normal in structure, though somewhat dilated. Spencer obj. 16 mm. Compensating ocular 9×.

## PLATE 24.

FIG. 2. Rat 9. Uranium; killed on sixth day. The entire cortex and the outer stripe of the outer zone of the medulla are shown. In the upper two-thirds of the figure are seen the normal divisions I of the proximal convoluted tubule filled with carmine granules in rod-like formations, which show as an intense black in the photograph, and which obscure all details in the structure of these tubules (Div. I). A small area of round cell infiltration is seen at A, and below this is a group of regenerated tubules with deeply staining large nuclei and dense protoplasm (Reg. Div. III). To the left of this group is a glomerulus, which separates the cortex and the outer stripe of the outer zone of the medulla, with its heavily granulated division I, and below are seen many tubules whose epithelium is entirely destroyed and whose lumen is filled with homogeneous detritus (Div. III). Spencer obj. 16 mm. Compensating ocular 9×.

## PLATE 25.

FIG. 3. Rat 7. Bichromate; killed on fifth day. Similar view of cortex and medulla as is in figure 2. In the upper quarter of the section surrounding the intact glomeruli are seen the divisions I of the proximal convoluted tubules with their epithelium destroyed and their lumen filled with detritus (Div. I). In the medullary ray which extends diagonally across the middle of the figure are seen sections of the thick ascending limb of the loop of Henle with pycnosis of the nuclei and desquamation of the epithelium. In the outer stripe of the outer zone of the medulla are seen the well preserved divisions III which appear darker in the photograph, due to the presence of the carmine granules which they contain (Div. III). Spencer obj. 16 mm. Compensating ocular 9×.

## PLATE 26.

FIG. 4. Rat 9. Uranium; killed on fifth day. The figure is taken from the area of severest degeneration, *i. e.*, the outer stripe of the outer zone of the medulla, where marked regenerative changes are clearly seen. At A is a tubule whose lumen is almost entirely filled with a syncytium-like mass of protoplasm with large vesicular nuclei. Similar large deeply staining nuclei are seen in many of the tubules. At B is a typical giant-cell with its rounded projection into the lumen of the tubule. Spencer obj. 16 mm. Compensating ocular 9×.

FIG. 5. The tubule B of figure 4 is shown. At the lower end of the section of the tubule is seen a rounded mass of protoplasm which contains six enlarged deeply staining nuclei. On both sides of this giant-cell are seen large nuclei with their surrounding protoplasm which, by growth extension, are creeping along the bare membrana propria. Above the giant-cell is a degenerated cell, out of focus, which obscures the lumen at this point. Spencer obj. 4 mm. Compensating ocular 9×.

## PLATE 27.

FIG. 6. Detail of figure 7. The tubule shown is the one in the center of figure 7, lying between the long horizontal degenerated tubule which is filled with detritus, and the oval-shaped collecting tubule. The infiltrating leucocytes are seen to be composed of both polymorphonuclear leucocytes and lymphocytes. At the lower edge of the infiltrated tubule is seen a densely staining row of fusiform proliferating tunica propria cells. Spencer obj. 4 mm. Compensating ocular 9 $\times$ .

FIG. 7. Guinea pig 4. Uranium; killed on ninth day. Across the middle of the figure stretches a row of tubules whose epithelium is entirely destroyed and whose lumen is filled with light staining granular detritus. Scattered throughout the sections are tubules whose lumens are filled with leucocytes, and in other places these leucocytic infiltrations occur between the tubules. There is also marked proliferation of the tunica propria cells, which aid in the formation of the densely staining areas of cellular accumulation. Spencer obj. 16 mm. Compensating ocular 9 $\times$ .

## PLATE 28.

FIG. 8. Guinea pig 4. Uranium; killed on ninth day. In the center of the section is a large tubule filled with light staining detritus. Extending upwards from the lower end of the section of the tubule is a strand of fusiform fibroblasts, which scatter out irregularly throughout the detritus. In one place beginning fibril formation by these cells is seen. On the right is a deeply staining strand of proliferating tunica propria cells and a glomerulus. Spencer obj. 4 mm. Compensating ocular 9 $\times$ .

## PLATE 29.

FIG. 9. Guinea pig 40. Uranium; killed on twenty-first day. Same region as figures 1 and 10. The glomeruli are completely enclosed in the mass of proliferating fibrous tissue and atypical regenerated epithelial cells, and there is marked cystic formation in their capsular spaces. The shrinkage of the connective tissue has lessened the area of changed kidney tissue considerably, and these rays of fibrous tissue now extend to the capsule. The collecting tubules on each side of this area are normal in structure and somewhat dilated. Spencer obj. 16 mm. Compensating ocular 9 $\times$ .

FIG. 10. Guinea pig 42. Uranium; killed on ninth day. The figure is taken from the lower layer of the cortex adjacent to the medulla. Throughout the section are dark, intensely staining strands which consist of proliferated tunica propria cells around the collapsed tubules. The contrast of the diffuse proliferation of the connective tissue as contrasted with the later stages is well shown. Casts are seen in the collecting tubules, and there is some dilatation of these tubules. The glomeruli are normal. Spencer obj. 16 mm. Compensating ocular 9 $\times$ .

FIG. 11. Rat 7. Bichromate; killed on fifth day. In the center of the figure is a group of tubules with deeply staining large nuclei and rather dense protoplasm. In places there is an excessive number of nuclei and they are seen heaped upon each other in irregular layers. The tubules surrounding these regenerated segments are practically normal. Spencer obj. 16 mm. Compensating ocular 9 $\times$ .

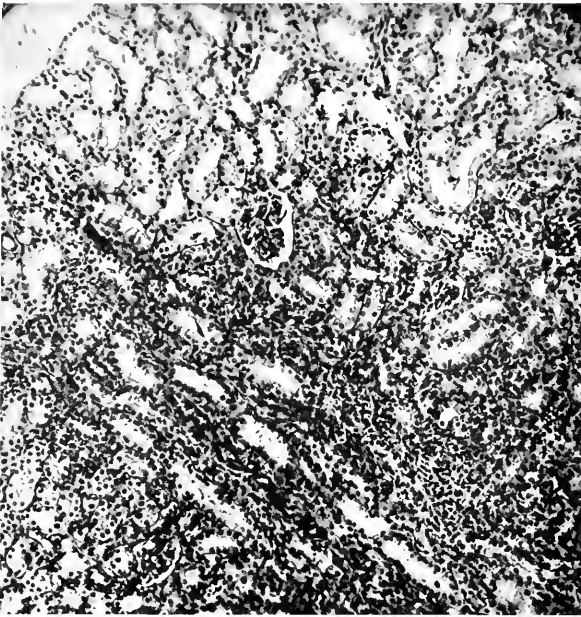
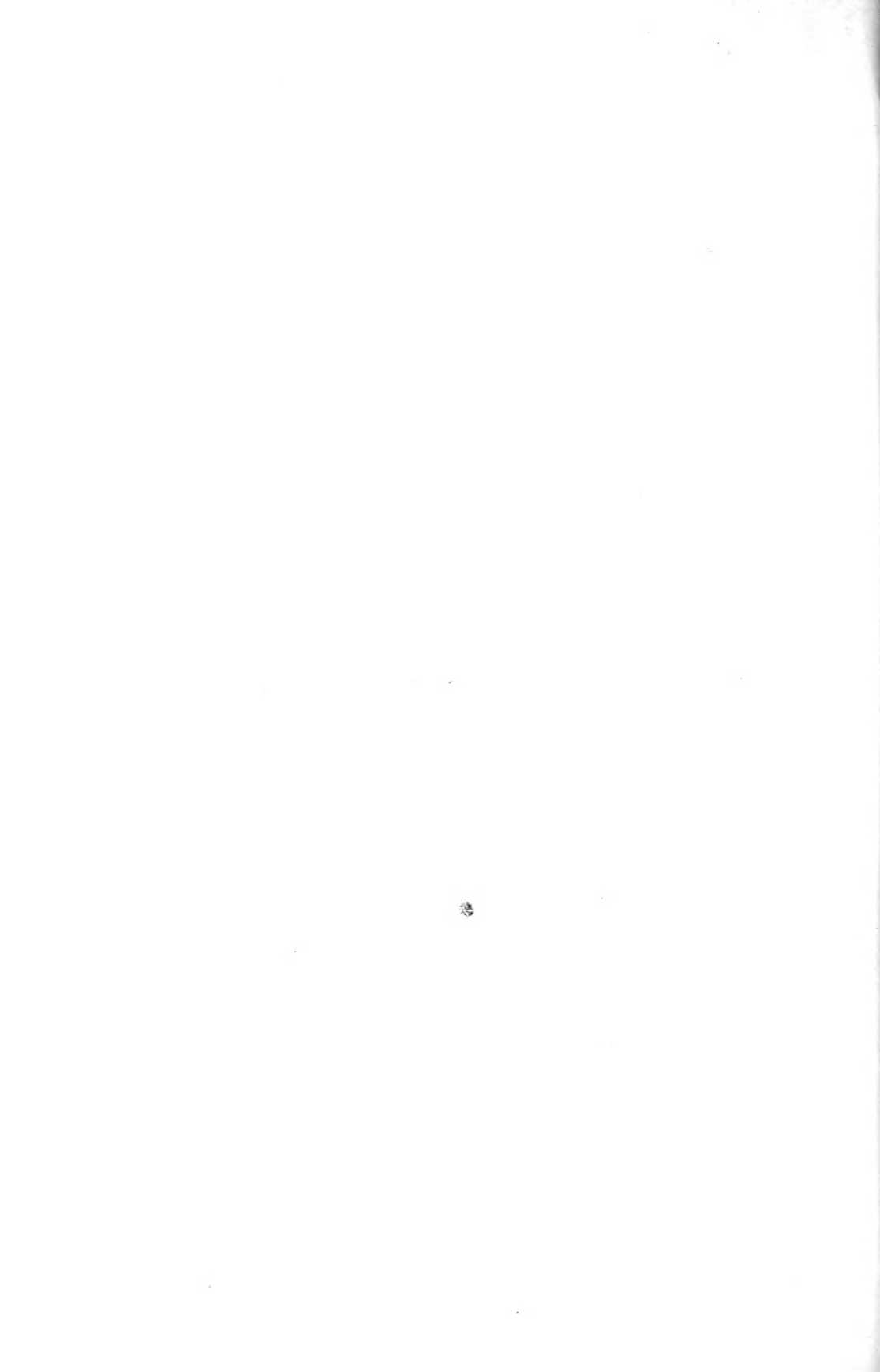


FIG. 1.

(Oliver: Histogenesis of Chronic Uranium Nephritis.)



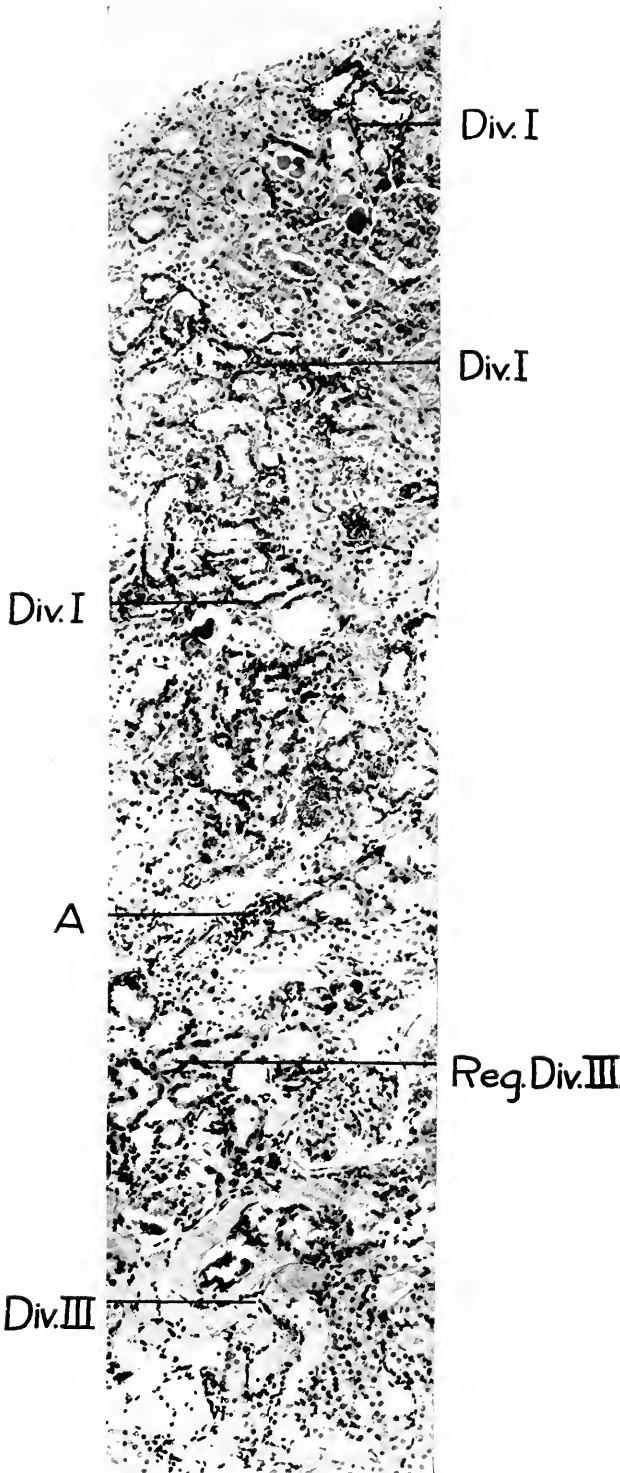


FIG. 2.

(Oliver: Histogenesis of Chronic Uranium Nephritis.)



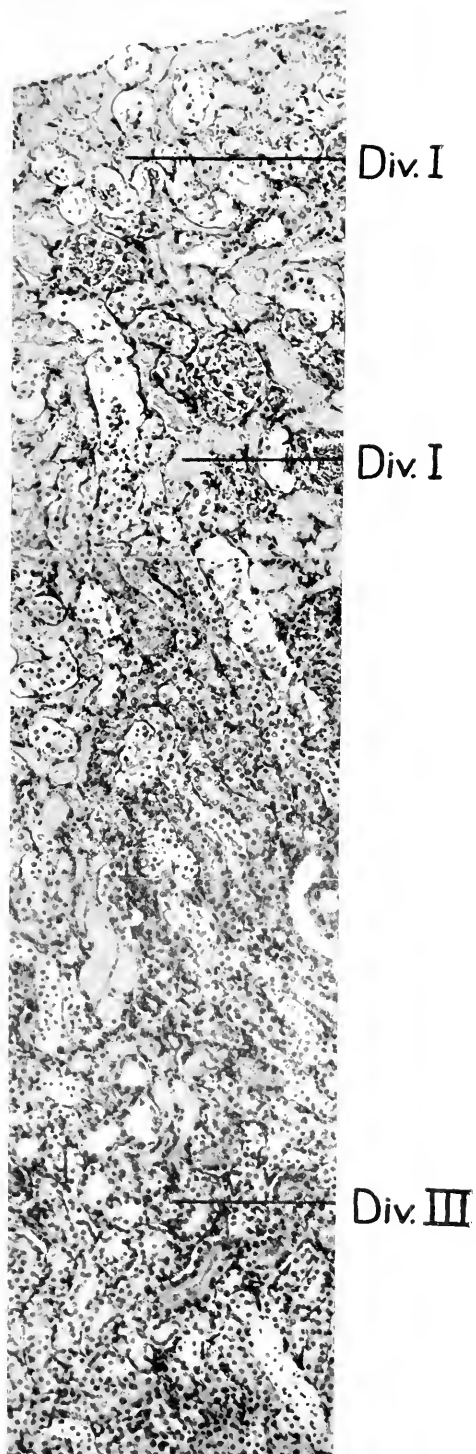


FIG. 3

(Oliver: Histogenesis of Chronic Uranium Nephritis.)





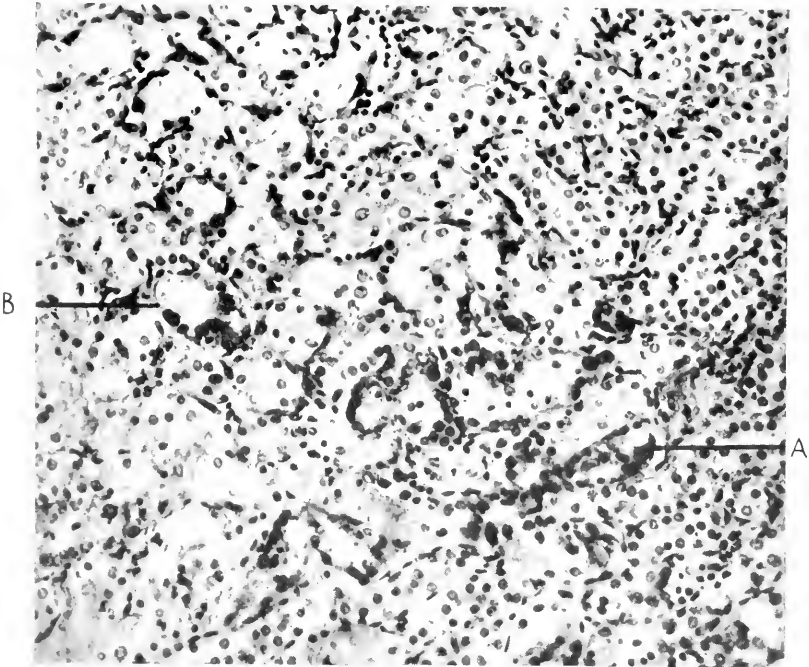


FIG. 4.

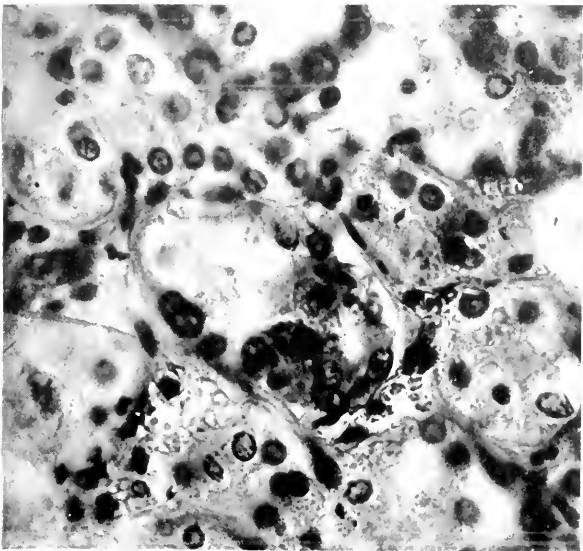


FIG. 5.

(Oliver: Histogenesis of Chronic Uranium Nephritis.)



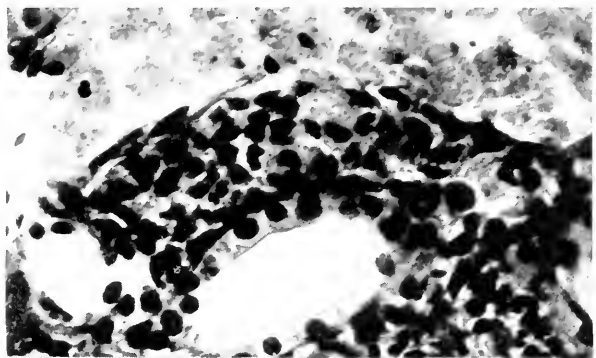


FIG. 6.

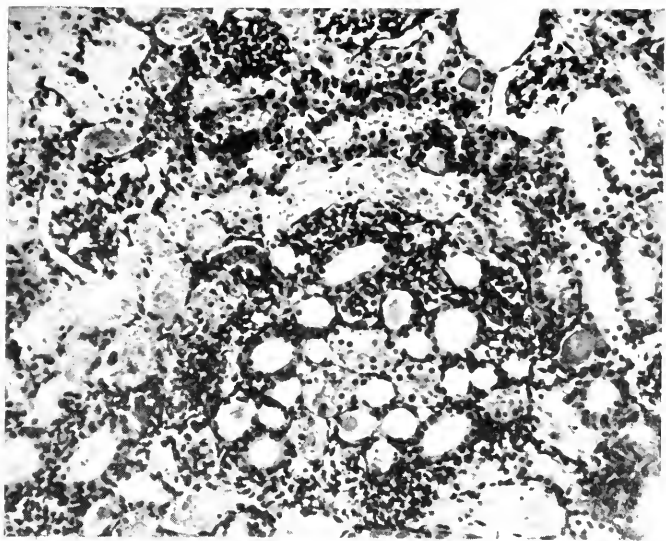


FIG. 7.

(Oliver: Histogenesis of Chronic Uranium Nephritis.)



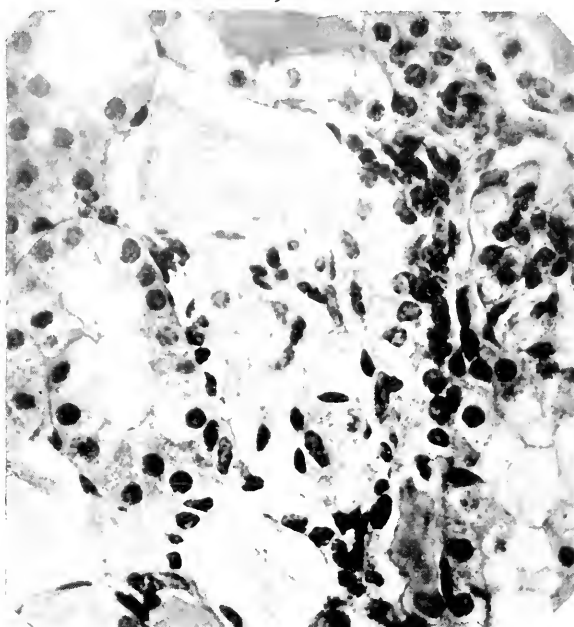


FIG. 8.

(Oliver: Histogenesis of Chronic Uranium Nephritis.



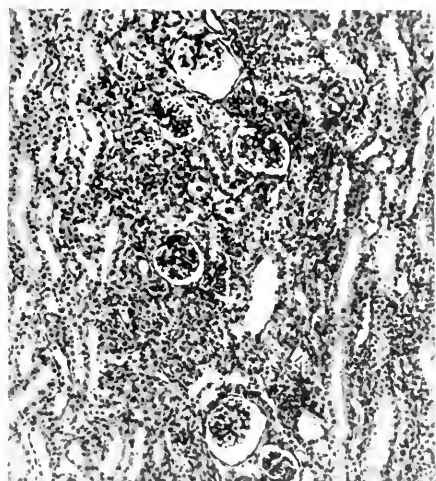


FIG. 9.



FIG. 10.

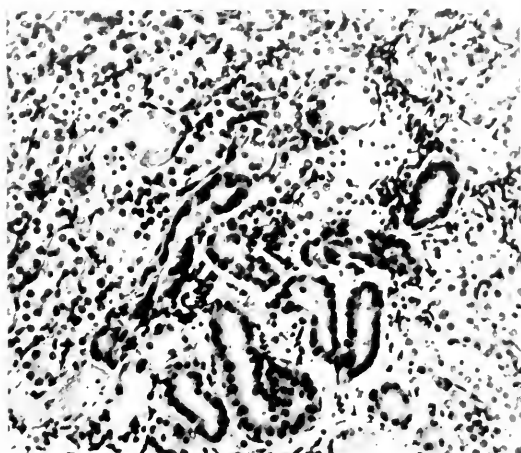


FIG. 11.

(Oliver: Histogenesis of Chronic Uranium Nephritis.)





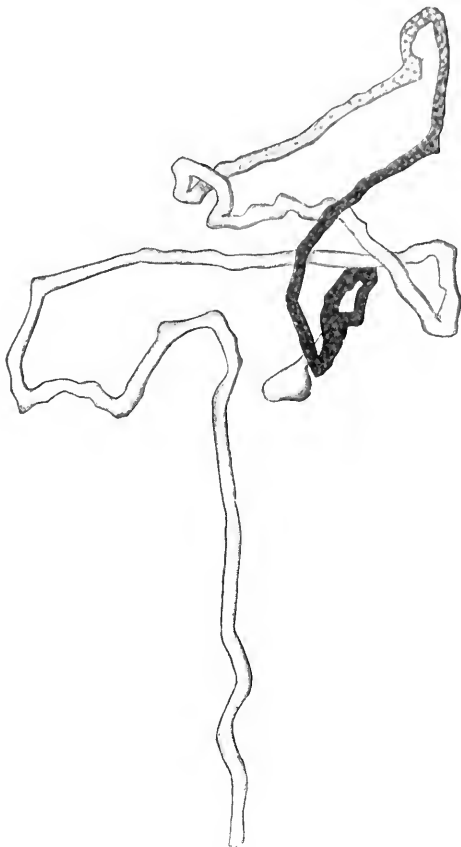


FIG. 12.

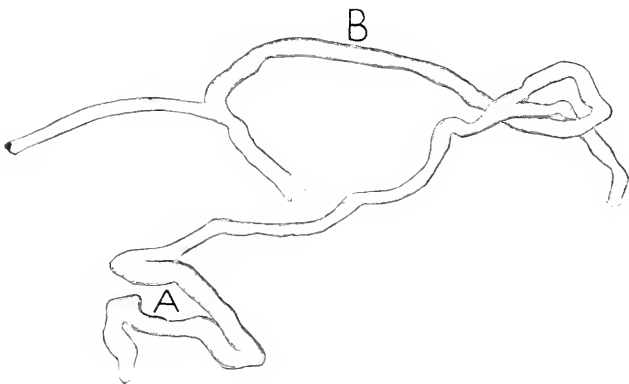


FIG. 13.

(Oliver: Histogenesis of Chronic Uranium Nephritis.)



## PLATE 30.

FIG. 12. Normal vitally stained rabbit (trypan blue). The proximal convoluted tubule with the upper part of its medullary portion is shown. That part of the tubule lying adjacent to the glomerulus is heavily impregnated with vitally stained granules, so that the unstained nuclei stand out in contrast as lighter areas. The number of granules decreases as one departs from the glomerulus, appearing as isolated groups and finally disappearing completely. Camera lucida drawing of isolated tubule. Bausch and Lomb obj. 1. Ocular 2/3.

FIG. 13. Normal vitally stained rabbit. The second convoluted tubule is seen at (A), connecting with the collecting tubule (B). The second convoluted tubule, as shown in Peter's illustrations, consists of but few loops as compared with the proximal convoluted tubule, and contains no vitally stained granules. Camera lucida drawing of isolated tubule. Bausch and Lomb obj. 1. Ocular 2/3.

## OBSERVATIONS ON THE ETIOLOGY OF GOITRE IN BROOK TROUT.

### IV. THE EFFECT OF FEEDING WITH FRESH AND STALE LIVER.\*

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This report includes (1) a brief summary of the sixth annual inventory of the state of the thyroid glands in the brook trout at the hatchery of the Blooming Grove Hunting and Fishing Club, and (2) an account of some experiments in the feeding of fresh and stale liver.

The arrangement and number of the ponds and troughs, the water supply, the strain of brook trout, and their distribution in the ponds and troughs according to age, have remained unchanged since our first observations in 1909. The crowding has gradually increased during these years, but the general external appearance of cleanliness of the ponds and troughs has not changed. Those containing the fry have always been unusually well cared for. The food, consisting of hog's liver and heart, has remained constant during the six years for all fish up to the ninth month of life, while for the past three years all fish over nine months old have been fed with hashed sea fish.

Histological examinations of the thyroids of a complete series representing specimens from all the ponds and troughs have been made yearly. The condition of the thyroid up to the time the change of food is made, *i. e.*, the ninth month of life, has not varied noticeably during these six years. All have shown marked active thyroid overgrowth, as noted in previous papers. When the food is changed to sea fish at the ninth month, the thyroid overgrowth is arrested, and the gland returns to its colloid or resting stage in about thirty-five to forty days (1). During the three years that this effect has been studied, no further hypertrophy or growth of the thyroid has been observed, although the fish remain in the ponds for a period of about two years after the change of food is instituted. On the other hand, when liver was used as the food throughout their lives in captivity, the thyroid overgrowth progressed continuously to visible external manifestations in practically all the fish by the end of the second year. The substitution of sea fish as a food has proved to be a specific curative and preventive measure under apparently the same conditions where liver as food caused continuous thyroid overgrowth.

\* Received for publication, February 24, 1915.

In previous papers (2, 3, 4) it has been suggested that the volume of water, its oxygen supply, its content of excreta (overcrowding), and the highly artificial food (liver and heart muscle) might be factors in causing the thyroid overgrowth. It was on account of the rapid regression of the thyroid overgrowth when sea fish was fed, and on account of its continuous growth when liver and heart muscle were fed, that food was more particularly suspected of playing an important part in stimulating the thyroid to this continuous growth. If this was a factor, it seemed probable that simple experiments in which the freshest liver was fed and experiments in which distinctly stale liver was fed might reveal differences in the thyroid growth. To this end the following experiments were made.

Two troughs were selected from the series of twenty-one. The fry of one were fed in the usual manner (twice daily) with the freshest liver, while the fry of the others were fed with portions of the same hashed liver as above, which had been kept in the cold chamber (11° to 15° C.) for two days. The experiments were begun on July 16 and terminated September 17, at which time the food of all fry was changed to sea fish. Two fish were taken at weekly intervals from each trough. As controls, specimens were taken from the twenty-one troughs at the beginning of the experiment, and two specimens at weekly intervals from one adjoining trough, which were fed on the general stockroom supply. At the end of the two months there were no gross changes in size, activity, or general appearance in the two sets. Histological preparations were made from all these thyroids. Study of the condition of the thyroids showed that there was a slight gradual increase in the degree of thyroid overgrowth noticeable in the second month in those fed with the freshest liver over those fed with the same liver held for two days. No difference could be distinguished between those fed with the freshest liver and the controls fed from the general supply.<sup>1</sup>

<sup>1</sup> Similar experiments have been carried out on rats, where better control could be had. Thus two series of twenty-one young rats each were divided into groups of three each. The first group was fed on the fresh hog's liver, from animals killed the same day; the second group was fed with the same amount of the same liver one day old; and each subsequent group was fed with the same liver one day older than the preceding group. It was kept at room temperature screened from flies. They were fed with liver six times weekly, while bread and water were kept continuously in the cages. All gained in weight, those getting the freshest liver slightly more than those getting the staler liver. One rat in each group was killed at intervals of two weeks, and the thyroids were examined microscopically. There was distinct hypertrophy, as judged by the reduction in stainable colloid and increase in the size of thyroid cells, in those groups getting liver 1, 2, and 3 days old, while those getting fresh liver and liver 4, 5, and 6 days old had normal or nearly normal glands.

These findings were the reverse of what I thought might occur, as I had in mind the possibility that autolysis and bacterial digestion of the liver might produce substances capable of stimulating the thyroid cells to increased activity.

A plausible explanation for these findings is not at hand. The lack of control over the amounts of liver taken by the fish seemed suggestive, but experiments with rats where the quantity was controlled show in general the same results. The idea that certain products of autolysis and bacterial decomposition of the liver act as irritants to the thyroid may be abandoned. Since controlled experiments show only a very slight thyroid hypertrophy, one may conclude that the diet is only a contributing factor, and that it may act by increasing the work of the thyroid in order to maintain a general increase in metabolism, especially in connection with the overfeeding of a nutritionally incomplete diet.

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# THE ORIGIN AND STRUCTURE OF A FIBROUS TISSUE WHICH APPEARS IN LIVING CULTURES OF ADULT FROG TISSUES.\*

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PLATES 31 TO 36.

## INTRODUCTION.

Within the past few years a considerable amount of investigation has been done upon the cultivation of living tissues outside the body, and various phases of the subject have been studied. Thus far, however, it appears that there has been very little consideration of the reactions that occur in the plasma clot of the tissue cultures. Harrison,<sup>1</sup> among others, has shown that a supporting framework of some sort is necessary in the medium in which the living tissue is imbedded in order to obtain a prolific growth and movement of the tissue cells. The fibrin net which is present in plasma or lymph cultures forms this framework. If the plasma or lymph be defibrinated and the resulting serum used as a medium in which to cultivate the tissues there is, in general, comparatively little growth or movement of the tissue cells. In this paper<sup>2</sup> the results of experiments with living cultures of adult frog tissues are presented, which show that a definite reaction frequently occurs in the plasma clot. As a result the fibrin net present in the clot gradually loses its typical structure and becomes transformed into a new fibrous tissue closely resembling in its appearance, structure, and function various types of white fibrous connective tissues.

The thanks of the author are due to Professor Ross G. Harrison

\* Received for publication, March 4, 1915.

<sup>1</sup> Harrison, R. G., The Reaction of Embryonic Cells to Solid Structures, *Jour. Exper. Zööl.*, 1914, xvii, 521.

<sup>2</sup> A report of this work was given at the meeting of the American Society of Zoölogists held at Philadelphia, December 31, 1914.

for his suggestion of the problem and for his invaluable assistance and advice from time to time as the work progressed.

#### MATERIAL AND METHODS.

In the work reported in this paper, the tissue cultures have all been made by the hanging drop method, and the usual precautions to keep the cultures sterile have been observed. This method of tissue culture has become quite well known within the last few years, and a full description of the technique will not be given in this paper.<sup>3</sup> The plasma used has been secured in the following manner: An anesthetized frog is opened ventrally and the heart exposed. A hypodermic syringe (a five cubic centimeter glass Luer with a short fine needle is very satisfactory) is then inserted in the ventricle. If the frog has not been anesthetized too heavily, a considerable amount of blood (one to two cubic centimeters) can then be drawn out of the ventricle. When all the blood that can readily be secured—it may take some minutes—is obtained, the syringe is withdrawn and the blood centrifuged. In the present experiments it has been the rule to use only fresh plasma. In most cases both the plasma and the tissue for imbedding in it were obtained from the same animal. The tissue taken from the animal was placed in Ringer's solution and cut into small pieces.

During the course of the experiments it was found necessary to preserve and section a great many of the preparations, and in order to do this it was necessary to get the plasma clot with the imbedded tissue free from the cover-glass without injury. To accomplish this it was found convenient to coat the cover-glasses before being used with a thin coat of celloidin. This was done by immersing the cover-glasses in a weak solution and then letting them dry. The celloidin solution is sterile but if, for any reason, there is need of

<sup>3</sup> The reader is referred for further details regarding technique and for a general survey of the field to papers by the following authors: Harrison, R. G., *The Outgrowth of the Nerve Fiber as a Mode of Protoplasmic Growth*, *Jour. Exper. Zööl.*, 1910, ix, 787. Carrel, A., and Burrows, M. T., *Cultivation of Tissues in Vitro and Its Technique*, *Jour. Exper. Med.*, 1911, xiii, 387. Oppel, A., *Causal-morphologische Zellenstudien. V. Mitteilung. Die aktive Epithelbewegung, ein Faktor beim Gestaltungs- und Erhaltungsgeschehen*, *Arch. f. Entwicklungsmechn. d. Organ.*, 1912, xxxv, 371.



sterilizing the cover-glasses it can be done in a dry heat. The celloidin will stand a temperature of 100° C. without apparent change. Using these celloidin-coated cover-glasses it becomes a comparatively simple matter to get the plasma clots with the imbedded tissue free from the cover-glass. In general the clot will remain attached to the cover-glass in the killing fluid and through the washing and dehydrating up to the absolute alcohol, at which point the celloidin will be dissolved and the preparation will, in most cases, free itself from the cover-glass with no injury whatsoever. The preparation can then be imbedded and sectioned in the usual manner. Sections of the preserved preparations were cut at eight microns. Various methods of staining the preserved cultures have been tried and a description of these is given in a later section of this paper.

#### STUDY OF THE LIVING CULTURES.

As shown in the accompanying table (table I) a total of 996 cultures have been made from various tissues of the adult frog during the course of these experiments, and of this number 846 have been under observation and form the basis for the results reported in this paper. It is possible by a study of the living preparations to follow closely the reactions that occur in the plasma clot, and photographs

TABLE I.  
*Summary of the Results of the Experiments.*

Material imbedded.	Total No. of cultures made.	No. of cultures rejected.	No. of cultures studied.	No. of cultures in which fibers appeared.
Liver.....	476	81	395	86
Spleen.....	170	50	120	55
Muscle.....	115	19	96	37
Dead tissues and corpuscles...	135	None	135	0
Living blood corpuscles.....	30	None	30	3
Starch grains.....	20	None	20	0
Blank clots.....	50	None	50	0
Total.....	996	150	846	181

of the various stages can be made while the cultures are alive. The reactions that occur in the clot appear to be the same with any of the tissues tried, and the description given will suffice for any of the preparations. The plasma in these tissue cultures, as is well

known, clots within a few minutes after the piece of living tissue has been added, and the imbedded tissue is firmly held by the fibrin net which forms in it. This fibrin net is of the same character as would be formed whenever plasma clots, and when observed under the microscope shows a typical fibrin net structure. This condition of the fibrin net continues in a high percentage of the preparations during the entire history of the culture (table I). Growth may occur in the imbedded tissue and the cells move freely out into the clot and yet no transformation of the fibrin net take place. The tissues of the mature frog are very inert as compared with the embryonic tissues of various animals, and in many cultures very little or no growth or movement of tissue cells occur. The heart muscle tissue of the frog forms, as a rule, quite a definite exception to the above statement, and in most cultures of this tissue a considerable cellular growth and movement will occur. However, whether there has been any growth of the imbedded tissue or not, some of the cultures in the course of two or three days will show that a change is taking place in the plasma clot surrounding the tissue. In the region lying nearest to the imbedded tissue, small fiber-like structures appear. At first these are quite small and fairly indistinct. They are of the same color as the fibrin net but have a different index of refraction, and they are large enough to be easily seen with the low power of the microscope. These fiber-like structures increase in size and number and ramify in all directions through the clot. They generally reach a high state of development by about the fifth to seventh day after the making of the preparation. Their development, however, will continue after this until in many cases at the end of two weeks almost the entire plasma clot has been changed into a new tissue which has a very different appearance and structure from that of the typical fibrin net. So far as has been observed, and a number of the cultures have been kept alive for periods exceeding three weeks before they were preserved, these fibers which form in the plasma clot are permanent. The fibers are consolidated to the greatest extent near the imbedded piece of tissue and radiate from that region in all directions to the periphery of the clot. To one studying the fiber formation in the living cultures it appears that they are direct outgrowths from the imbedded tissue. Experiments

which are given later in this paper show that such is not the case, however, and that the fibers are formed from the elements of the fibrin net. The clots in which the fibers have formed become very firm and solid and very unlike in this respect an unchanged plasma clot in which the typical fibrin net is present. This fact is particularly evident when one tries to remove a clot from the cover-glass for permanent preservation. The clots in which the fibers have formed can be handled roughly without injuring them at all. The regular plasma clots are very tender and have to be handled with the greatest care when they are removed from the cover-glasses in order to prevent their serious injury.

Microphotographs have been made of a number of the living cultures to show different stages of the fiber formation. Figure 1 is a microphotograph of a culture of liver tissue at a magnification of 33 diameters taken when the culture was three days old. A few of the small, newly formed fibers are to be noted in the plasma immediately surrounding the upper edge of the tissue. All the fibers present in this culture are not seen, inasmuch as it was impossible to bring them all into focus. This preparation, in the size and number of the fibers present, is typical of the average condition found in a two to three day culture in which the reaction has taken place. Numerous examples, however, of still greater development have been observed. It has been found that the development of the fibers is more rapid and prolific with spleen tissue than it is with any other of the tissues that have so far been used. Figures 2 and 3 are both microphotographs of spleen cultures taken when they were four days old. The magnification is 27 diameters in both instances and the photographs show clearly how striking and prominent the fibers are in the living preparations. Numerous blood corpuscles can be seen scattered through the plasma. Figure 4 is a microphotograph of a nine day culture which has been magnified 40 diameters and gives one a clearer view of the network of fibers which has formed in the plasma clot. It can also be noted that around the edge of the imbedded tissue a specialized part of the plasma has developed and the fibers appear to arise from this region. Such a region is not present in the clot at first and the early fibers appear to arise from the edge of the imbedded tissue as shown in

figure 1. This region, in the older cultures, in which there is a transformation of the plasma, always occurs first in that region of the clot which is in direct contact with the imbedded tissue, and the changes which take place would therefore appear to be due to some action of the living tissue. The cultures from which figures 2 and 4 were taken were preserved, and a description of them is given later in this paper. In the cultures which are shown in figures 1 to 4 very little or no growth of the tissue cells took place, and, as has been noted, such is generally the case with most of the adult frog tissues. There are exceptions, however, and cultures have been under observation in which cell growth and movement as well as fiber formation could be noted. Such a condition is shown in figure 5, which is a photograph of a ten day liver culture. In this preparation a considerable number of fibers are to be noted in the plasma and others were present which are out of focus. To the left and below, it can be seen that a considerable outgrowth of cells from the tissue has occurred, among which are numerous large spindle cells. No relation is to be observed between these cells and the fibers. In cultures of heart muscle tissue a different condition is sometimes to be observed. An example of this is shown in figure 6, which is a photograph taken from a ten day culture of heart muscle tissue. In this culture quite a heavy fiber formation took place in the plasma surrounding the imbedded tissue during the first three or four days. Later spindle cells moved out from the tissue and followed the course of these fibers. This is the condition shown in the figure and several instances can be noted of the stereotropism of these cells. A similar condition has been observed in other cultures of this same tissue.<sup>4</sup>

#### STUDY OF THE PRESERVED CULTURES.

In order to make a histological examination of the fibers and their relations both to the imbedded tissue and the plasma clot, it was necessary to make and study mounted sections of the preserved cultures. A description of the methods used in preparing this material for microscopic study has already been given in a previous section of this paper.

In figure 7, which is a microphotograph of a spleen culture pre-

<sup>4</sup> Harrison, *Jour. Exper. Zööl.*, 1914, *loc. cit.*

served at the end of the second day, an early stage in the fiber formation is to be seen. The fibrin net is for the most part unchanged, but a number of distinct fibers have been formed. These have arisen from the region of the clot lying nearest the tissue. Here the typical fibrin net structure has been partly lost, and it appears that there has been a consolidation or fusion of the fibrin elements to form a fibrous tissue. The evidence from the histological study of all the cultures apparently shows that the fibers have arisen through a transformation of the fibrin elements. However, the connection between the imbedded tissue and the fibers is so close in a great many instances that it is impossible to say from these observations alone that the fibers are not outgrowths of the imbedded tissue. Experiments, which are given in the following section of this paper, confirm the histological study of the cultures and make it certain that the new fibrous tissue arises from a transformation of the fibrin net. Figure 8 shows a microphotograph of a six day culture of spleen tissue at a magnification of 67 diameters. Figure 2, above, shows this same culture as it appeared while living at the time when it was four days old. In the two days intervening before it was preserved a considerable development of new fibers occurred, so that in figure 8 many more fibers are to be seen. This preparation is considered typical of a six day culture, and is worthy of careful consideration. The fiber formation shows a great advance over that pictured in figure 7. It will be noted that the fibrin net in the immediate vicinity of the imbedded tissue has been changed from the typical fibrin net structure into a dense, heavily staining tissue composed of wavy fibrous bundles in which, with sufficient magnification, the individual fibrils can be seen. From this dense fibrous area of the transformed fibrin net, bundles of fibers extend in all directions through the plasma clot. These bundles are heavier nearer the tissue and gradually grow smaller as they proceed out to the periphery and are finally lost in the undifferentiated fibrin net. They unite with other similar bundles of fibers at various places throughout their length. This condition can be seen in figure 9, which is a photograph of a portion of this culture magnified 667 diameters. In the upper part of this figure can be seen the dense fibrous tissue which lies next to the imbedded tissue, and proceeding from this area the wavy fibrous

bundles can be seen running to the less differentiated areas of the clot. These bundles can be seen to anastomose and interweave with each other to a considerable degree. In figure 10, which is also a photograph of a portion of this same culture magnified 1,000 diameters, the individual wavy fibrils which form the bundles can be seen. It is quite a difficult matter to show clearly the detailed structure of the fiber formation in the clots by means of microphotographs, especially with the higher magnifications. Accordingly in figure 11 a drawing of the same portion of this culture as is depicted in figure 10 is shown, which makes the structure of the transformed fibrin net still clearer. In the lower part of the figure a part of the imbedded tissue can be seen. In close contact with it is shown the heavy fibrous tissue with the bundles of fibers. These run to the periphery of the clot, gradually decreasing in size as they leave the region of the imbedded tissue, and are finally lost in the untransformed fibrin net. At the ending of the bundles, as well as in various other places shown in the drawing, an atypical arrangement of the fibrin elements can be noted. Apparently there is a fusion of these elements to form the fibrils and a grouping together of the fibrils to form the fibrous bundles. With the magnification used in this drawing ( $\times 667$ ) it is possible in many cases to trace the fibrils from their point of origin in the fibrin net into the fibrous bundles. The fibrin net in the region of the imbedded tissue has become almost completely transformed from its typical structure, and only small areas of the clot remain which are unchanged. In the outlying regions of the clot large areas of the untransformed fibrin net can be seen. The complete absence of all isolated tissue cells is to be noted in this region of the culture.

Another preparation of interest is shown in figure 12. This microphotograph is from a nine day culture of spleen tissue. In figure 4 is shown a photograph of this culture made while living and just before its preservation. The fiber formation in this culture did not begin as early as it usually does and the development of the fibers is not as far advanced as found in some of the younger cultures as, for example, in figure 8, which is a six day culture. In figure 12 attention should be called to the part of the plasma clot which is lying in contact with the imbedded tissue. In this area the

fibrin net has become so changed from its typical structure and takes a stain so much like the tissue, that in the photograph it is difficult to make out the exact boundary line between the clot and the imbedded tissue. This transformed area is similar to that shown in figure 8, but the transformation is more complete. From the central part of this area great bundles of fibers are given off which run to the periphery of the clot. The greater portion of the clot in this culture still retains its typical net structure.

If conditions remain favorable the fiber formation continues in the plasma clot until practically the whole area has become transformed into a new fibrous tissue. The fibrin net disappears and the clot in some of the older cultures appears as a reticular tissue. The preparation shown in figure 13, which is a photograph at 100 diameters, presents an example of such a case. This culture has two pieces of liver tissue imbedded in the clot and was fourteen days old when preserved. The appearance shown in this figure is not exceptional, and a number of other preparations have been made which show as advanced a condition of fiber formation. It can be noted that practically the entire clot has become changed into what appears to be a reticular tissue. Figure 14, which is a microphotograph of a portion of this culture at 667 diameters, pictures one of the least differentiated areas, and from this it can be seen that the clot has been almost completely changed in its structure. In figure 17 a drawing of a portion of this preparation may be seen which shows the structure of the transformed fibrin net more clearly than is possible with photographs. At both the upper and lower edges of the drawing are shown a few of the cells of the two pieces of imbedded tissue. The clot lying between these two pieces of tissue has almost completely lost its characteristic fibrin net structure and instead we find a new fibrous tissue containing a great many bundles of wavy fibers. To all appearances this new fibrous tissue is identical with true reticular tissue as pictured and described by Mall.<sup>5</sup> It is held by many authorities that the fibrils of reticular tissue are identical with the fibrils of areolar tissue.<sup>6</sup> In some cases it can even be demonstrated

<sup>5</sup> Mall, F. P., Reticulated Tissue, and Its Relation to the Connective Tissue Fibrils, *Johns Hopkins Hosp. Rep.*, 1893, i, 171, plates 17 and 18.

<sup>6</sup> Schäfer, E. A., Text Book of Microscopic Anatomy, in Quain, J., Anatomy, 11th edition, London and New York, 1912, ii, pt. 1, 123, 124, figure 203.

that the fibrils of reticular tissue are in direct continuity with the white fibrils of the adjacent areolar tissue. In other words, the fibrils composing a reticulum may join together to form the bundles of fibers in the nearby areolar tissue. Such a relation between the fibrils and the fibrous bundles is also found in the tissue cultures where, as shown for example in figures 11 and 17, the fibrous bundles are composed of a great many fibrils, and in many instances these fibrils can be traced to where they leave the bundle and join the fibrin net. The bundles of fibers are typical of those found in areolar tissue. They grow smaller as they get away from the imbedded tissue, and in the outlying regions we find them breaking up and the fibrils of which they are composed continuing to form a typical reticular tissue.

#### THE ORIGIN OF THE FIBERS.

Although the study of the prepared cultures gives evidence that the new fibrous tissue arises through a transformation of the fibrin elements of the clot, nevertheless the close connection existing in the cultures between the imbedded tissue and the bundles of fibers makes it impossible to say that they have not arisen as direct outgrowths of the imbedded tissue. In order to settle the matter definitely it was found necessary to make several series of experiments with special types of cultures.

In the first series a large number of cultures were made in which the living tissues were imbedded in defibrinated serum instead of plasma. The method used in this series was to take the plasma from the frog by the usual technique and to divide it into two parts. One part was used in making the regular type of plasma cultures with living tissues imbedded. The other part of the plasma was defibrinated and used in making other similar cultures. The two sets of cultures were identical in every way except for the fact that some had the plasma while the others had the defibrinated serum, and these latter owing to the absence of the fibrin could not form any clot around the imbedded tissue. The results obtained from these experiments have always been the same, namely, the serum preparations have always failed to show any fiber formation, while in many of the parallel cultures in which the plasma was used the transformation of the



fibrin net occurred. Such a result indicates that the fibers are formed by a transformation of the fibrin net and not as outgrowths of the imbedded tissue.

In another series of experiments preparations were made as usual with plasma, but no tissue was imbedded. The plasma will clot of itself shortly after having been exposed to the air, and it was thought worth while to determine if fibers would form in these blank clots in which no tissue was present. Blank cultures were also made in which the plasma was coagulated by the addition of an extract obtained from fresh muscle tissue. This extract was obtained by mashing muscle tissue of the frog in a small amount of Ringer's solution and then centrifuging it to get rid of the blood corpuscles and small pieces of the tissue. By adding a drop of this extract to the plasma a very firm clot can be had in a few minutes. All the cultures of this series gave negative results and no transformation of the fibrin net occurred.

In a third series of experiments plasma cultures were made as usual, except that either living blood corpuscles, blood corpuscles which had been killed by heating, or starch grains were imbedded in place of living tissues. In the cultures in which the dead corpuscles and the starch grains were imbedded negative results were obtained and no change of the fibrin net occurred. On the other hand, in three of the thirty cultures in which the living blood corpuscles were imbedded a transformation of the fibrin net occurred. In figure 15 is shown, at a magnification of 100 diameters, a microphotograph of a culture in which living blood corpuscles were imbedded without any tissue. It can be noted that in the region in which the corpuscles are present quite a complete transformation of the fibrin net has occurred and a new fibrous tissue with bundles of long wavy fibers has been formed. A study of the fibers reveals no apparent difference in structure from those formed when living tissue is imbedded in the plasma. The outlying regions of the clot still maintain the typical fibrin net structure. The fact then that the transformation of the fibrin net can occur and the fibers arise in cultures in which only living blood corpuscles have been imbedded shows definitely that the fibers are not formed as outgrowths of the imbedded tissue, but they have been formed through a transformation of the fibrin net.

This result confirms that obtained from the histological study of the prepared cultures, which is given above.

#### FACTORS INVOLVED IN FIBER FORMATION.

The experiments just mentioned in the previous section showed that the new fibrous tissue arose by a transformation of the fibrin net. They also indicated that this transformation was caused by the presence of living tissues or cells in the plasma, inasmuch as cultures in which starch grains or dead blood corpuscles were imbedded, as well as the blank clots, all failed entirely to show any transformation of the clot. It was deemed wise to experiment further in an endeavor to find out if the transformation of the clot was dependent upon the presence of living tissues. To do this several series of parallel cultures were made use of. In order to make the method of experimentation clear it may be well to give the complete data of one such series of cultures. On February 14, 1914, the following cultures were made in the same manner from the same plasma and under identical conditions:

- 10 cultures of living muscle tissue.
- 10 cultures of living liver tissue.
- 13 cultures of living spleen tissue.
- 10 cultures of dead spleen tissue (the tissue had been heated to 60° C. in order to kill it).

Two days later fibers began to appear in the plasma clots of a number of the muscle and liver cultures, and fibers were also present in eleven out of the thirteen living spleen cultures. In the preparations in which the spleen tissue had been killed by heating no fibers ever appeared, and the plasma clots of these cultures never showed any change whatever from the fibrin net structure. Six parallel series of cultures, which were essentially the same as the series just described, have been made from time to time during the course of the work and the various dead tissues which were imbedded have been killed both by heating and poisoning. The results have always been the same, namely, no transformation of the fibrin net has ever occurred in any of the cultures in which the dead tissues were imbedded, while the reaction has always been present in some of the parallel living tissue cultures. In some of the preparations in which

the dead tissues were imbedded the extract from fresh muscle tissue was used to coagulate the plasma. The addition of the tissue extract did not in any apparent manner change the result previously obtained and no fiber formation has occurred in the clot of any culture except those in which either living tissue or blood corpuscles were imbedded. Attention should be called to another line of evidence which is in harmony with the above, and that is that in the living cultures in which the fiber formation takes place, the fibers appear first in the part of the clot which is in contact with the living imbedded tissue, and this part of the clot always shows the most complete transformation. In some cases the outlying regions of the clot, which are considerably removed from the influence of the tissue, retain the fibrin net structure while a complete transformation of the clot may take place near the imbedded tissue (figure 12). The results from all the experiments strongly suggest that the reaction which causes the fiber formation is due to some property of the living tissues which have been imbedded. The author, however, does not regard the tests so far made as exhaustive enough to say with absolute certainty that the transformation of the clot can only take place under the influence of living tissues or cells. There is the possibility that the reaction is one which will occur under the influence of other conditions which as yet are not known. Whether or not such is found to be the case the essential thing to be noted is that the imbedded living tissues are by themselves able to bring about a radical transformation of the fibrin elements of the clot and the consequent formation of a new fibrous tissue which, in appearance at least, is a typical connective tissue.

It has been found possible by mechanical means, such as pulling and twisting the clots with dissecting needles, to cause the fibers to form very rapidly in the clots in which the living tissues have been imbedded.<sup>7</sup> In such cases the piece of living tissue is placed in the

<sup>7</sup> An account of some early work by Leo Loeb along this line that is suggestive is given by Adami, J. G., *The Principles of Pathology*, 2d edition, Philadelphia and New York, 1910, i, 40. To quote: "When a drop of uncoagulated lymph is placed between two glass slides, the mere act of pulling one slide over the other leads to the appearance of fibrils, which grow in length and bulk; which, like those of connective tissue, are not only intracellular, but actually traverse cell bodies situated in their path; which show themselves first in imme-

drop of plasma as usual. After the clot has formed around the imbedded tissue the formation of the fibers can be aided by manipulating the clot with needles and thereby exerting tension in various directions. Such a preparation is shown in figure 16 in which a piece of spleen tissue was imbedded and the clot manipulated with needles. The culture was then sealed as usual, left over night, and preserved the next morning. It will be noted that the fibers are very irregular and formed in the various regions following the path of the needles through the clot. Such a condition is different from that shown in the previous figures in which the fibers were allowed to develop slowly in an undisturbed clot. In either case the structure of the fibers appears to be the same and to have resulted from a consolidation or fusion of the elements of the fibrin net. In the disturbed clot these processes are evidently hastened by the introduction of the mechanical factor. Many cultures have been made during the course of the work in which the clots were disturbed. Also the effect has been noted of imbedding killed tissues and then disturbing the clots. So far such cultures have always failed to show the fiber formation except that, if the plasma is caused to clot by the addition of a little of the fresh tissue extract and then the clot is disturbed, there occasionally appears to be an attempt at fiber formation. Possibly it might be expected that such a result would be obtained, since it is probable that the fresh tissue extract contains the constituents of the living tissue which are responsible for the transformation of the clot.

Two points may be noted as a result of these experiments: first, the evidence at hand strongly indicates that the presence of living tissue or of living cells is necessary to cause a transformation of the plasma clot; and, second, that when either of these is present the introduction of a mechanical factor may aid in a more rapid formation of the fibers.

The fact that mechanical means could be used to assist in the formation of the fibers suggested to the author the possibility that diate connection with these cells, the cells, as we now hold, liberating an enzyme that determines the modification of the more soluble protein into a precipitated or coagulated modification. But the lines of the precipitation are evidently along the lines of strain."

the fibers in the regular cultures were due to the action of living cells which had moved out from the imbedded tissue or that were present in the plasma; in other words, that perhaps the fibers marked the paths of the various isolated cells which had become separated from the tissue or of cells which were present in the plasma, such as blood corpuscles, lymphocytes, etc. In most of the cultures it can readily be observed that numerous blood cells are present in the plasma clots. In actively growing cultures also a great many cells move from the imbedded tissue to various regions of the clot. The cultures of heart muscle tissue of the adult frog show this to the greatest advantage, and in such cultures many instances can be noted in which the fibers are in close relation to the isolated spindle cells. Such a culture is shown in figure 18, which is a microphotograph at 100 diameters of a four day culture. Numerous spindle cells can be seen in the fibrin net to the right of the imbedded tissue. The path of these cells in their movement from the tissue to their present position can be traced by the appearance of the fibrin net. In such a culture it appears probable that the fibers which have been formed have arisen partly at least as a result of the action of the cells in forcing their way through the fibrin net. In the cultures of heart muscle tissue which have been under observation the formation of fibers is generally associated with the spindle cells which have moved through the clot from the imbedded tissue, whereas in cultures of spleen and liver tissue in which no growth or movement of tissue cells has taken place the fibers arise directly from that region of the plasma clot which is in contact with the imbedded tissue. A comparison of figure 8 with figure 18 will make this statement clear. In the first figure with spleen tissue, the fibers have arisen in close connection with the imbedded tissue, while in figure 18, with the heart muscle tissue, the fibers have, in general, arisen in close contact with the individual cells that have wandered out from the tissue, and there are very few fibers which are in contact with the imbedded tissue. In figure 19, which is a microphotograph at 667 diameters of a portion of figure 18, a better idea can be obtained of the relations existing between the spindle cells and the fibrous bundles in these cultures of heart muscle. The figure shows one of the spindle cells moving through the plasma clot. Below its path can be traced by

the appearance of the fibrin net which has been transformed into a fibrous tissue. Long fibrous bundles also radiate out laterally from the region of the cell. Above the fibrin net still retains its typical structure. In figure 20, which is a microphotograph of an eight day heart muscle culture at a magnification of 667 diameters, a condition is shown which is typical of that found in the older cultures of this tissue. The part of the clot lying next to the imbedded tissue and through which the cells have wandered has become almost entirely transformed into the bundles of fibers and with the cells lying between these a tissue is formed which is entirely similar, in its appearance at least, to the regular connective tissue of the frog. It is generally supposed that the fibroblasts which wander into a fibrin clot from the injured tissues during wound healing digest the fibrin and then form the new connective tissue fibers intracellularly. Such, however, is not the case with these fibers which have formed from the elements of the fibrin net. They are not digested by the tissue cells which have wandered into the clot, but, on the other hand, they are acting as permanent fibers and have combined with the tissue cells to form a connective tissue.

In the cultures of heart muscle tissue, then, the results indicate that the tension exerted by the isolated spindle cells in their movements through the plasma clot plays a part in the fiber formation. However, it can be shown that the movements of isolated tissue cells are not responsible for the formation of fibers in all the cultures, for the same reaction will take place in cultures in which there are no isolated cells present in the clot, and, therefore, the transformation which occurs must be due to the action of the living tissue as a whole without the aid of any apparent mechanical factor. In cultures of adult frog spleen and liver there is, in general, very little, and in some cases no growth or movement of tissue cells, and yet it is in cultures of these tissues that the most prolific fiber formation takes place. With care it has been found possible to make a culture in which the plasma is also free from blood corpuscles. Such a culture, providing there is no outgrowth of cells from the imbedded tissue and that a transformation of the fibrin clot has occurred by which the new fibrous tissue has been formed, demonstrates that the imbedded tissue can bring about the reaction without the aid of iso-

lated cells. A number of such instances have been noted during the course of the work and a microphotograph of such a culture is shown in figure 13, in which preparation the clot showed an almost complete absence of isolated cells and no movement of tissue cells occurred. Notwithstanding the absence of isolated cells in the clot an almost complete transformation of the fibrin net took place. Such a result makes it clear that the imbedded living tissue is, without the aid of isolated cells or of any other apparent mechanical factor, able to bring about the formation of the fibrous tissue from the fibrin elements of the clot.

#### THE NATURE OF THE NEW FIBROUS TISSUE.

In an endeavor to determine the nature of the new fibrous tissue, which arises in the cultures through a transformation of the fibrin elements of the clot, experiments with various specific stains have been made. Also the new tissue has been subjected to digestion and acid tests. It was hoped that these experiments would show definitely whether or not the fibers composing the new tissue were fibrin in character, although apparently identical in their appearance, structure, and function with the regular collagenous fibers of connective tissue.

*Staining Reactions of the New Fibrous Tissue.*—Two stains have been made use of, both of which are supposed to be specific to differentiate between fibrin and connective tissue; namely, Mallory's connective tissue stain modified according to Mall,<sup>8</sup> and Van Gieson's picrofuchsin stain. Early in the work it was found that with the Mallory stain the fibers formed in the fibrin net were stained a blue color which very closely resembled the color of the regular connective tissue fibrils when stained in the same manner. With this stain fibrin is supposed to stain red in contrast to the blue of the connective tissue. However, the fibrin of frog plasma stains a purplish blue instead of a red with this stain. The differentiation, though between the purplish blue of the undifferentiated fibrin net and the almost ultramarine blue of the new fibers, is very clear. All the microphotographs that are shown in this paper were made from

<sup>8</sup> Mall, F. P., On the Development of the Connective Tissues from the Connective-Tissue Syncytium, *Am. Jour. Anat.*, 1901-02, i, 338.

preparations which were stained with the Mallory stain, and from these it can be seen that the differentiation is very sharp between the fibrin net and the fibers which form in it. In comparison with the regular connective tissue fibers which can be studied in the imbedded tissues of the cultures it can be said that with the Mallory stain there is in most instances very little difference in color between them and the fibers which have been formed by the transformation of the plasma clot. The regular connective tissue fibers stain a perfect ultramarine blue, while the fibers which form in the clot in the same culture are generally a little darker in color and a little less clear. In short it may be said that when this stain is used the regular connective tissue fibers and the fibers formed in the clot stain so nearly the same that there is no real difference in their appearance. This result, however, is not obtained when the cultures are stained with Van Gieson's stain. This stain colors connective tissue fibers red and other tissues yellow. Using this stain on the preparations it has, thus far, been found impossible to obtain any differentiation between the undifferentiated plasma clot and the fibers formed in it, the entire clot with the fibers taking a yellow stain. The fibers can be clearly seen in the clot of a culture thus stained, but they have the same yellow color as the parts of the clot which have not been transformed, in distinction to the red color of the regular connective tissue fibers. With this stain, therefore, the evidence is that the fibers formed in the clot retain their fibrin character. A combination of the two stains has been used in a number of instances. In this case the culture is stained first with the Mallory stain, then partially decolorized with acid alcohol, and restained with Van Gieson. Preparations treated in this manner show a clear differentiation between the fibers in the clot which stain a light green, as do also the regular connective tissue fibers in the imbedded tissue, and the remainder of the clot which takes a pinkish stain.

*Digestion and Acid Tests with the New Fibrous Tissue.*—The fact that fresh fibrin is easily dissolved by digestion with pancreatin or by the use of a weak acid, whereas connective tissue fibers are not so affected, was used as a basis for determining the nature of the fibers formed in the clot. The method of experimentation was as follows: In a culture in which the fibers were present in the clot, the cover-



glass, to which were attached the clot and the imbedded tissue, was removed and immersed in the pancreatin solution at 37° C. or in a dilute solution of acetic acid. Cultures ranging in age from five to fourteen days have been experimented with in this manner. The result, with either of the tests, has always been that within a few hours the entire clot containing the fibers completely disappears leaving the imbedded tissue free. Such a result apparently indicates that the fibers formed in the clot remain fibrin in character. The tests, however, are not conclusive, because of the intimate relations existing between the fibrin net and the fibers which form in it. The destruction of the fibrin net either by digestion or with a weak acid naturally causes a breaking down of the fibrous bundles which are formed in it and a scattering of the individual fibrils, inasmuch as they are not attached to the imbedded tissue and therefore their only support is the fibrin clot in which they are formed. Thus far it has proved to be a difficult matter to get a test that can be used in these tissue culture preparations that will definitely distinguish between fibrin and connective tissue fibers. Mall<sup>9</sup> mentions the great difficulty he found in using the digestion and acid tests in the study of the early differentiation of the connective tissue fibers. He states that the results from various tests contradicted each other, so that he hesitated to draw any conclusions from them. Difficulties of the same kind have been encountered in this work, and the author does not lay great weight upon the results of the tests so far made as showing definitely whether or not the fibers formed are really connective tissue fibers.

In brief, the structure and function of the new fibrous tissue and the appearance with Mallory's stain all indicate that it is a true connective tissue. On the other hand, the Van Gieson stain and the acid and digestion tests give evidence that the new fibrous tissue retains the fibrin character of the fibrin net from which it formed. Work along various lines is in progress and it is hoped that as a result the true character of the new fibrous tissue can be definitely settled.

<sup>9</sup> Mall, *Am. Jour. Anat.*, *loc. cit.*, p. 339.

## DISCUSSION.

With the results of these experiments at hand it may be well to discuss briefly the significance of the changes which take place in the cultures of adult frog tissues. It is evident that in the reaction which occurs in the plasma clot, involving as it does a transformation of the fibrin net into a new fibrous tissue, we have a process which must be fundamental in wound healing. A wound under the most favorable conditions for healing becomes quickly filled with the fluid plasma and lymph which pour into it from various sources. This quickly coagulates and a fibrin net is formed.<sup>10</sup> Under the influence of the living injured tissues, and aided to some extent by the tension exerted on it through the movements of the tissues, this mass of fibrin soon loses its typical structure. As shown in this paper, the fibrin elements fuse or consolidate to form fibrils, and these unite to form long wavy fibrous bundles which freely intertwine and anastomose and ramify through the entire clot. In a short time there has arisen, in place of the fragile and easily destroyed plasma clot, a new fibrous tissue which is tough and resistant and which functions for the time, at least, as a regular connective tissue. The transformation of the clot also causes a shrinkage, and the wound space is thereby lessened, the edges of the wound are held firmly in place, and a support is formed for the outgrowth of the tissue cells.

From the work presented in this paper it also appears that in wound healing, at least, there is good reason for believing that this new fibrous tissue which arises by a direct transformation of the fibrin clot, without intracellular action, remains as a permanent connective tissue. Hertzler<sup>11</sup> has already put forth such a view which he bases on results obtained from wound healing. He shows

<sup>10</sup> For a summary of the part played by fibrin in wound healing, see Marchand, F., *Der Process der Wundheilung*, Stuttgart, 1901, 52-55. The fact that the fibrin elements in wound healing consolidate to form fibers is also shown; see Marchand, *loc. cit.*, pp. 245-255, figures 64-67.

<sup>11</sup> Hertzler, A. E., Pseudoperitoneum, Varicosity of the Peritoneum and Sclerosis of the Mesentery. With a Preliminary Note on the Development of Fibrous Tissue, *Jour. Am. Med. Assn.*, 1910, liv, 351; The Development of Fibrous Tissues in Peritoneal Adhesions, *Anat. Rec.*, 1915, ix, 83.

that the fibrous bundles are present in the clots in wound healing and also that they will take a specific connective tissue stain a short time after they are formed. Hertzler believes that the fibers arise through some chemical action. Although there is considerable support for the view that the connective tissue fibers, in general, arise through intercellular action,<sup>12</sup> the idea put forth by Hertzler that they arise by a transformation of the fibrin elements of the clot does not appear to have received any support. In the present paper it has been possible to show by the use of tissue culture methods that under the influence of living adult frog tissue a transformation of the fibrin elements of the clot may give rise to a new fibrous tissue which in appearance, when stained with Mallory's stain, structure, and function is identical with regular connective tissue. The experiments which have so far been made use of in an endeavor to determine the nature of the new fibrous tissue have failed to settle the question definitely, and until it is possible to get a test that will determine the question no definite statement regarding the nature of the tissue should be made. The work with the older cultures shows that the new fibrous tissue remains permanently (figure 17), and also that it is not digested by the tissue cells which wander into it, but, on the other hand, the two combine to form a typical areolar tissue (figure 20). There appears to be no reason for believing that the fibers of the new tissue would ever be replaced by other fibers formed intracellularly. Personally, from the study of the living and preserved cultures that has been made, the author feels that there is good ground for the belief that the fibers which have formed in the clot through a transformation of the fibrin elements are identical with those found in areolar and reticular tissues. Whether or not the work which is now being carried on, which embraces a study of the origin of the connective tissue fibers in wound healing and also in embryonic development, bears out this belief, as suggested above, a direct transformation of the clot into the new fibrous tissue such as has here been definitely shown must be a process which is of the highest value in wound healing.

<sup>12</sup> For an account of the various views held regarding the origin of the connective tissues, see Schäfer, *loc. cit.*, pp. 116-122. Adami, *loc. cit.*, p. 429.

## SUMMARY.

In living cultures of various kinds of adult frog tissues, which have been made according to the hanging drop method, there occurs, in many cases, a transformation of the plasma clot by which it becomes entirely changed from a typical fibrin net both in appearance and structure.

The changes in the fibrin net generally begin when the culture is from two to three days old. During these changes it appears that the elements of the fibrin net fuse or consolidate, and as a result a great number of fine wavy fibrils are formed which unite to form wavy bundles of fibers, and these freely intertwine and anastomose as they ramify through the area of the plasma clot. The transformation of the fibrin net occurs first in the region of the clot which lies next to the imbedded tissue, gradually extends to the distal regions of the clot, and in time—as a rule in about two weeks—the entire plasma clot becomes changed from the fibrin net into a structure which to all appearances is identical with regular connective tissue. Photographs of both living and preserved cultures have been made to show the course of the transformation of the plasma clot and the development of the fibers.

Experiments have been made which show that the fibers which are formed are not outgrowths of the imbedded tissue. Also they are not formed by an intracellular action, but arise directly by a transformation of the fibrin elements of the plasma clot.

Experiments have been made which indicate that the transformation of the fibrin net will not occur unless it has come under the influence of living tissues or of living isolated cells. However, mechanical means, such as exerting tension on the clot with needles, may hasten the formation of the fibers. Also, in some cultures, movements of living isolated cells appear to aid in the formation of the fibers. The living tissues alone, however, are able to cause the fibers to form without the aid of any apparent mechanical factor. This is shown by cultures of various tissues in which no cell movement occurs and in which the plasma clot is undisturbed and yet a prolific formation of fibers may take place.

Experiments have been made in order to determine the true nature

of the transformed plasma clot and to see if the new fibrous tissue were still fibrin in character. The results that have so far been obtained from these tests have not been entirely conclusive and leave the question unsettled.

The transformation of the fibrin net results in a shrinkage of the clot. It also becomes very tough and resistant to injury and, therefore, entirely different from the fragile and easily destroyed fibrin net when in its original condition. It is believed that such a reaction must play an important part in wound healing. A study of the relation between connective tissue fibers formed in wound healing and in embryonic development to the fibers formed in the plasma clot is being made, and the results will appear in a later paper.

#### EXPLANATION OF PLATES.

All of the figures shown are microphotographs<sup>13</sup> with the exception of Nos. 11 and 17 which are drawings. Figures 1 to 6 inclusive were taken from the living cultures. The remainder of the figures were taken from the preserved cultures and were made with a Bausch and Lomb microphotographic apparatus equipped with a Zeiss microscope and apochromatic lenses.

#### PLATE 31.

FIG. 1. Three day culture of liver tissue.  $\times 33$ . From the upper edge of the imbedded tissue a number of small fibers can be seen projecting into the plasma clot.

FIG. 2. Four day culture of spleen tissue.  $\times 27$ . A prolific growth of fibers is shown. This culture was preserved. See figures 8 to 11.

FIG. 3. Four day culture of spleen tissue.  $\times 27$ . Two pieces of imbedded tissue are shown, from both of which a great many of the fibers are given off into the plasma. The pieces of tissue were not connected at first. Later they became connected by the fibrous formation in the clot and then cells wandered out on these fibers as shown in the figure. Numerous blood corpuscles are present in the clot. Also many small light areas are noticeable in the clot. These are the result of condensation on the glass slide.

FIG. 4. Nine day culture of spleen tissue.  $\times 40$ . Fibers are shown at a higher magnification. Note specialized area of the clot around the imbedded tissue from which the fibers arise. This culture was preserved. See figure 12.

FIG. 5. Ten day culture of liver tissue.  $\times 33$ . A considerable cellular movement from the imbedded tissue has occurred, as well as a prolific fiber formation.

FIG. 6. Ten day culture of heart muscle tissue.  $\times 33$ . A heavy cellular growth and fiber formation have occurred. At the lower edge of the tissue cells can be noted which are apparently travelling along the fibers.

<sup>13</sup> The author is glad of this opportunity to express his thanks to Professor Alexander Petrunkevitch for his advice and assistance in making some of the microphotographs.

## PLATE 32.

FIG. 7. Two day culture of spleen tissue.  $\times 100$ . An early stage in the fiber formation is shown in this figure. A small area of the clot which is in contact with the imbedded tissue is becoming transformed. The rest of the clot retains the typical fibrin net structure.

FIG. 8. Six day culture of spleen tissue.  $\times 67$ . A heavy fiber formation is to be noted in the clot. A dense fibrous tissue has been formed in the clot almost entirely around the imbedded tissue, and from this area the fibers radiate to the outlying regions. See figure 2; also figure 11 which shows a drawing of a part of this region.

FIG. 9. A part of the culture shown in figure 8.  $\times 667$ . A portion of some of the bundles of wavy fibers are shown and also some of the untransformed clot. Above can be seen a part of the dense fibrous tissue which surrounds the imbedded tissue.

FIG. 10. A part of the culture shown in figure 8.  $\times 1,000$ . The fine fibrils which compose the bundles of fibers can be noted, as well as some of the untransformed fibrin elements of the clot.

## PLATE 33.

FIG. 11. A drawing of a part of the culture shown in figure 8.  $\times 667$ . Below a small portion of the imbedded tissue is shown. Lying in close contact with this is the dense fibrous tissue which has formed in the clot. Bundles of fibers, which compose this area, radiate to the outlying regions of the clot, gradually grow smaller, and finally end in the untransformed fibrin net. The elements of the net appear to fuse together to form the fibrils which in turn unite to form the fibrous bundles. No isolated cells are present in this region of the culture.

## PLATE 34.

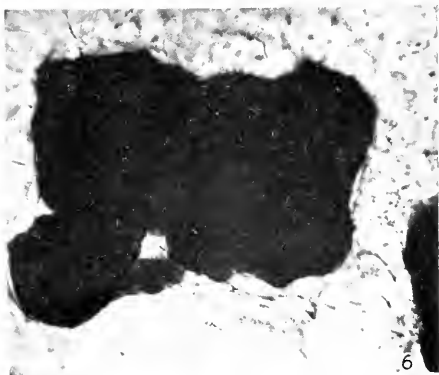
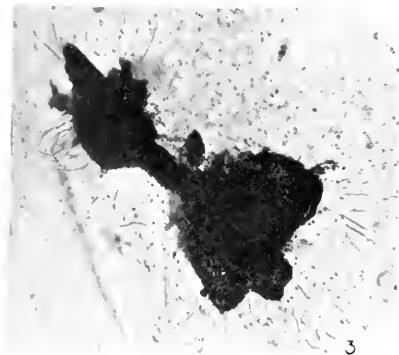
FIG. 12. Nine day culture of spleen tissue.  $\times 100$ . The portion of the clot lying in contact with the imbedded tissue has become changed from the fibrin net to a dense fibrous tissue from which large bundles of fibers are given off. The transformation has been so complete in this region that it is difficult to make out the boundary between the clot and the imbedded tissue. See figure 4.

FIG. 13. Fourteen day culture of liver tissue.  $\times 100$ . Two pieces of the imbedded tissue are shown. The portion of the clot lying between them has become almost completely transformed into what gives every appearance of being a reticular tissue. See figure 17, which shows a drawing of this same culture.

FIG. 14. A portion of the new fibrous tissue formed from the clot of the culture shown in figure 13.  $\times 667$ .

FIG. 15. A preparation in which living blood corpuscles were imbedded in place of a piece of tissue.  $\times 100$ . The fibers have formed in the region in which the corpuscles were imbedded.

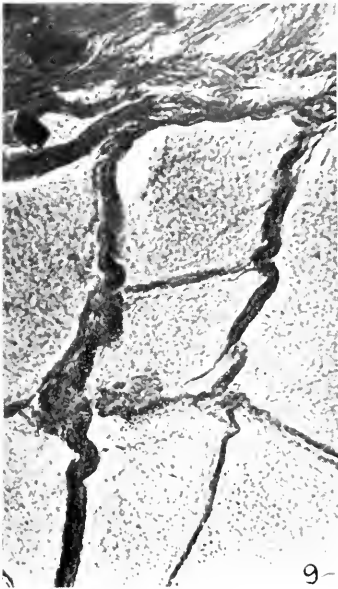
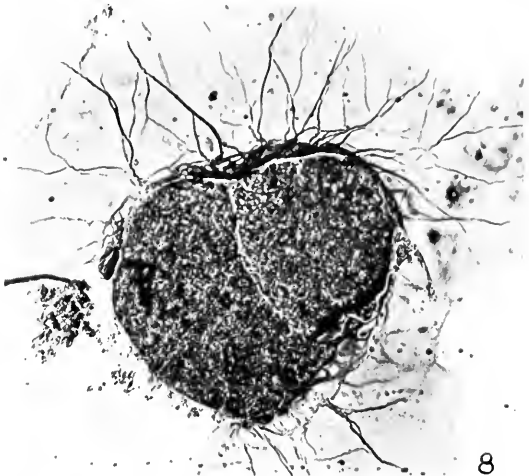
FIG. 16. A preparation in which the clot was disturbed by exerting tension with needles.  $\times 100$ .



(Baitsell: Fibrous Tissue in Adult Frog Tissue Cultures.)







(Baitsell: Fibrous Tissue in Adult Frog Tissue Cultures.)

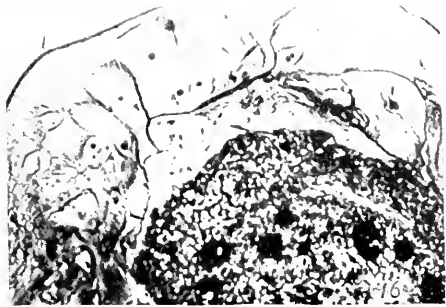
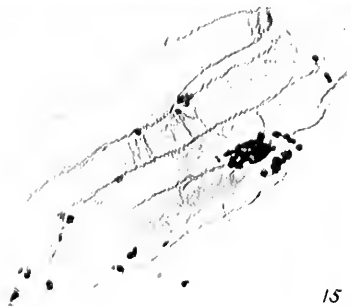
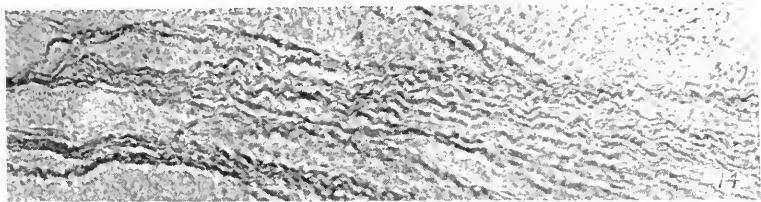




FIG. 11.

(Baitsell: Fibrous Tissue in Adult Frog Tissue Cultures.)





(Baitsell: Fibrous Tissue in Adult Frog Tissue Cultures.)



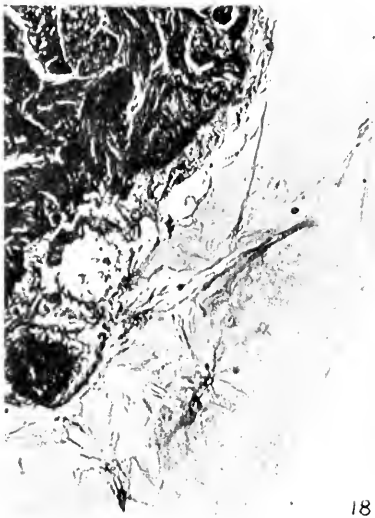


FIG. 17.

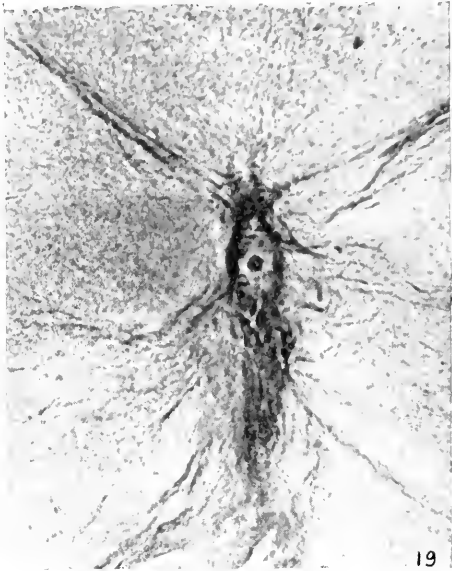
(Baitsell: Fibrous Tissue in Adult Frog Tissue Cultures.)



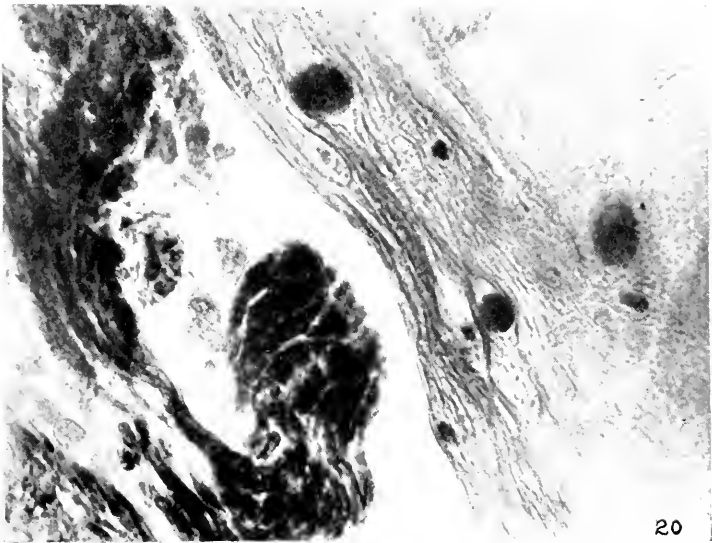




18



19



20

(Baitsell: Fibrous Tissue in Adult Frog Tissue Cultures.)



PLATE 35.

FIG. 17. A drawing of a part of the culture shown in figure 13.  $\times 667$ . It can be seen that practically the entire clot has been transformed into what appears to be a reticular tissue. Only very small areas are present in which a fibrin net appears. The most complete transformation of the clot lies nearest to the imbedded tissue.

PLATE 36.

FIG. 18. Four day culture of heart muscle tissue.  $\times 100$ . The fibers have formed in close connection with the cells which have wandered out from the tissue.

FIG. 19. A part of the culture shown in figure 18.  $\times 667$ . One of the spindle cells is shown. It has moved out from the imbedded tissue and its path can be traced by the appearance of the fibrin net, which has, in the vicinity of the cell, been largely changed into a fibrous tissue.

FIG. 20. A portion of an eight day heart muscle tissue culture.  $\times 667$ . The clot lying in contact with the imbedded tissue has become changed into the new fibrous tissue with its bundles of fibers, and these with the tissue cells which have wandered out from the imbedded tissue give a typical areolar tissue structure.

# THE NATURE OF ANAPHYLATOXIN.

## STUDIES ON IMMUNITY. II.\*

By J. BRONFENBRENNER, PH.D.

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It was shown by Friedberger (1) that the specific union between antigen, antibody, and complement is accompanied by the production of anaphylatoxin. If the mechanism of the Abderhalden reaction rests, as was suggested by my experiments (2), on the specific union of substratum and the specific fraction of immune serum, and if the presence of the complement is essential for the reaction to take place, there must appear during the reaction toxic products similar to those of Friedberger.

Experiments performed with this in view showed that the Abderhalden reaction is accompanied by the formation of highly active anaphylatoxin. These experiments were performed as follows.

The serum of three pregnant guinea pigs was mixed and placed in two centrifuge tubes, 6 c.c. in each, and about 3 gm. of boiled placenta tissue was added to

TABLE I.

Date.	Animal No.	Weight.	Dose.	Material injected.	Results.
Apr. 10...	160	250 gm.	3 c.c.	Supernatant fluid resulting from digestion of pregnant serum with placenta.	Death in 3 min.
Apr. 10...	162	255 gm.	2 c.c.		Death in 3 min.
Apr. 10...	163	255 gm.	1 c.c.		Death in 3 min.
Apr. 10...	165	260 gm.	0.5 c.c.		Grave symptoms. Recovery in 30 min.
Apr. 10...	167	250 gm.	2 c.c.	Supernatant fluid resulting from digestion of male serum with placenta (control).	No symptoms.
Apr. 10...	168	250 gm.	4 c.c.		No symptoms.
Apr. 10...	169	245 gm.	5 c.c.	Pregnant serum not having been digested with placenta (control).	Slight dyspnea.

\* A preliminary communication was presented at the meeting of the Pennsylvania State Medical Society, September 22, 1914. Received for publication, December 29, 1914.

one of them. At the same time 6 c.c. of the serum of a male guinea pig was put in another centrifuge tube with 3 gm. of boiled human placenta tissue. All these tubes were placed in the incubator for sixteen hours, and at the end of this time the tubes were centrifuged, the serum was separated, and tested for toxicity by intravenous injection into normal male guinea pigs of about 250 gm. The results are shown in table I.

Parallel with this series, the same sera, both pregnant and male, were tested by the regular Abderhalden method with the result shown in table II.

TABLE II.

Pregnant guinea pig serum.		Male guinea pig serum.	
Serum 1.5 c.c. Placenta 0.5 gm.	Serum 1.5 c.c. No placenta	Serum 1.5 c.c. Placenta 0.5 gm.	Serum 1.5 c.c. No placenta
16 hrs. at 37° C. in dialyzing thimbles.			
Ninhydrin test +	Ninhydrin test —	Ninhydrin test —	Ninhydrin test —

As the above experiments suggest, the appearance of dialyzable substances on which the Abderhalden reaction depends, and the toxicity of the serum resulting from the interaction between the serum and the placenta tissue in the test-tube run parallel, and must, therefore, depend on some specific mechanism very similar or even identical in both cases. Since, however, it was suggested by the experiments reported previously (3) that the dialyzable split products appear in the Abderhalden test as a result of autodigestion of the serum, the results of the above experiment suggest that the appearance of anaphylatoxin in this experiment may be due to autodigestion of the serum. This conclusion would open the question of the mechanism of the formation of anaphylatoxin in general.

There exist at present two main theories explaining the origin of anaphylatoxin: one is a chemical theory developed by Friedberger (1910-1914); the other, first adopted by Doerr (4), explains the phenomenon on a physical basis. In his earliest experiments Friedberger (5) obtained from normal guinea pig serum which had been allowed to stand for some time with the washed specific precipitate (formed by rabbit serum immunized against sheep serum with the serum of the latter), very strong poisons which killed the guinea pigs instantly with symptoms of acute shock. He named the poisonous substance anaphylatoxin, and assumed that it arose from the digestion of the specific precipitate by the ferments of normal guinea pig serum. Later it was shown by Friedberger (6) and his pupils (7) that the anaphylatoxin may be derived also from different bacteria and other proteins by their incubation with normal sera without concurrence of specific antibody, and that the physiological action of the poisons thus obtained is similar

to that of the chemical poisons obtained previously by Vaughan and Wheeler (8). But Friedberger obtained them from various proteins by the action of proteolytic ferments of normal serum, whereas Vaughan and Wheeler obtained them from the same proteins by means of chemical agents.

According to the physical theory, the source of anaphylatoxin is not the protein of the antigen but the serum itself, which becomes toxic as a result of the removal of some substances from it, by means of physical adsorption. Ritz and Sachs (9), for instance, assume that the normal toxicity of the serum is masked by the presence of antagonistic substances which may be removed by adsorption. The recent experiments of Bordet (10) and those of Nathan (11) and Muter-milch (12) in which the substances causing the formation of anaphylatoxin by incubation with guinea pig serum, namely, agar, starch, and kaolin, are not of a protein nature, seem to give strong evidence in favor of the physical theory. The question, however, is not yet settled, as Friedberger (13) objected to the results obtained with agar and starch on the basis that these substances contain a small percentage of protein impurities and thus still may furnish the substratum for digestion.

If the interpretation given to the results obtained in the experiment above is correct, that is, if the toxicity of the end-product in the Abderhalden test is due to the autodigestion of the serum, the findings of Muter-milch can be easily explained, since it was shown in my previous publication (3) that the addition to the serum of kaolin causes the autodigestion of the serum.

However, since Friedberger failed to confirm the findings of Muter-milch, it was deemed necessary to establish first whether the digestion of the serum with kaolin is followed by the appearance of anaphylatoxin.

TABLE III.

Date.	Animal No.	Weight.	Dose.	Material injected.	Results.
Apr. 20...	311	255 gm.	1.5 c.c.	Serum treated with kaolin and incubated at 37°C.	Grave symptoms. Found dead next morning.
Apr. 20...	312	250 gm.	1 c.c.	Serum treated with kaolin and incubated at 37°C.	Grave symptoms. Recovery in 15 min.
Apr. 20...	313	255 gm.	4 c.c.	Untreated serum.	No symptoms.
Apr. 20...	314	250 gm.	3 c.c.	Serum treated with kaolin immediately after removal from ice.	No symptoms.

Freshly drawn guinea pig serum was placed in a centrifuge tube and immediately after the addition of an excess of sterile kaolin the tube was placed on ice, and, as soon as the kaolin settled out, a new portion of kaolin was added. Special care was taken that the kaolin should not be lumped, and that the particles of kaolin should be dispersed as uniformly as possible in the serum before they

were allowed to settle. After the serum had been treated in this manner three times, which took over nine hours, the contents of the tube were centrifuged, the serum was separated, and transferred to the incubator. After sixteen hours' incubation the serum was injected intravenously into normal guinea pigs weighing about 250 gm., and was found to be toxic, as shown in table III.

Since kaolin is not soluble, it is evident that the toxicity of the supernatant fluid is due to changes in the serum. The possible objection that particles of kaolin may be suspended in this serum, and thus cause harm mechanically or chemically, is obviated by the fact that, as the experiment shows, the same serum, although toxic after incubation in the thermostat, is not toxic immediately after removal from kaolin.

As to the nature of the changes which may have taken place in the serum, my experiments show that the contact with kaolin deprives the serum of its antitrypsin and that subsequent incubation at 37° C. is followed by the autodigestion of the serum.

#### DOES PLACENTA FURNISH TOXIC PRODUCTS IN THE ABDERHALDEN REACTION?

In view of the results discussed above, the suggestion that the anaphylatoxin formed during the Abderhalden test originated from the serum seems to be strengthened. However, since in one instance the serum is digested with kaolin, which by itself cannot be the source of digestible material, whereas in the other instance serum is combined with a placenta tissue, and especially since it is asserted by Abderhalden that in these conditions of the experiment placenta is digested, it was deemed necessary to arrange the experiment so as to avoid even the possibility of the digestion of placenta before it would be possible to determine the source of the toxic material in this case. This was attempted in the following experiment (table IV).

12 c.c. of pregnant guinea pig serum were placed on ice in a tube with about 5 gm. of boiled human placenta tissue. Sixteen hours later the contents of the tube were centrifuged and the supernatant fluid<sup>1</sup> was separated from the sediment. 8 c.c. of this fluid were transferred into another tube and placed in the incubator (37° C.) for sixteen hours. At the end of this time the contents of

<sup>1</sup> Serum treated in this way, as was shown before, is deprived of its specific constituents as well as of the natural antitryptic inhibition, and if placed at this stage in the incubator it undergoes autodigestion.

### *Nature of Anaphylatoxin.*

the tube were tested for toxicity by the intravenous inoculation in normal male guinea pigs. The remaining 3 c.c. of supernatant fluid collected after centrifugation were tested for toxicity immediately after centrifuging.

TABLE IV.

Date.	Animal No.	Weight.	Dose.	Material injected.	Results.
Apr. 13...	200	250 gm.	3 c.c.	Supernatant fluid before incubation.	No symptoms.
Apr. 14...	202	253 gm.	3 c.c.	Supernatant fluid after incubation at 37 ° C. for 16 hrs.	Death in 2 min.
Apr. 14...	203	255 gm.	2 c.c.		Death in 2 min.
Apr. 14...	204	255 gm.	1 c.c.		Death in 2 min.
Apr. 14...	205	260 gm.	0.5 c.c.		Death in 5 min.
Apr. 14...	206	250 gm.	0.25 c.c.		Acute symptoms. Recovery in 15 min.
Apr. 14...	207	250 gm.	0.5 c.c.		Death in 3 min

As in the preceding experiment, here again the serum is not toxic immediately after the separation from placenta, but it becomes toxic in the absence of placenta if allowed to remain in the incubator, thus suggesting that the serum and not the placenta is the source of poison.

However, this conclusion is open to the criticism that the possibility of the serum containing minute particles of placenta in suspension after centrifugation is not definitely excluded. Such particles of placenta, although not resulting in toxicity of the serum immediately after removal from ice, may be digested later when the serum is transferred to the incubator, and thus contribute to the toxicity of the fluid. In order to show that such particles of placenta could not become the source of anaphylatoxin, the following experiment was undertaken (table V).

Pregnant human serum was placed in two centrifuge tubes, 5 c.c. in each, with about 2 gm. of human placenta protein. In two other centrifuge tubes pregnant guinea pig serum, 5 c.c. in each, was placed, again with 2 gm. of human placenta protein. Another exactly similar series of four tubes was set up with guinea pig instead of human placenta. All the tubes were placed for sixteen hours in the ice box, at the end of which time they were centrifuged and the serum was separated. One tube of each set was transferred into the thermostat for sixteen hours, to be injected subsequently into guinea pigs; the other tube of each set was tested immediately after the separation from the substratum.

If the toxic substances in the experiments above originated from



TABLE V.  
*Toxicity of Sera before Incubation.*

Date.	Animal No.	Weight.	Dose.	Material injected.	Results.
Apr. 17	211	265 gm.	2 c.c.	Human serum + human placenta	Death in 10 min.
Apr. 17	212	260 gm.	1.5 c.c.	Human serum + human placenta	Mild symptoms.
Apr. 17	214	250 gm.	1.5 c.c.	Human serum + guinea pig placenta	Mild symptoms.
Apr. 17	215	250 gm.	3 c.c.	Guinea pig serum + human placenta	No symptoms.
Apr. 17	216	248 gm.	4 c.c.	Guinea pig serum + guinea pig placenta	No symptoms.

*Toxicity of the Same Sera after Incubation at 37° C. for Sixteen Hours.*

Apr. 18	219	255 gm.	2 c.c.	Human serum + human placenta	No symptoms.
Apr. 18	220	250 gm.	3 c.c.	Human serum + human placenta	No symptoms.
Apr. 18	221	258 gm.	4 c.c.	Human serum + guinea pig placenta	No symptoms.
Apr. 18	226	255 gm.	1 c.c.	Guinea pig serum + human placenta	Death in 5 min.
Apr. 18	227	260 gm.	0.5 c.c.	Guinea pig serum + human placenta	Death in 5 min.
Apr. 18	228	260 gm.	0.25 c.c.	Guinea pig serum + human placenta	Coughing; recovery in 15 min.
Apr. 18	229	258 gm.	0.5 c.c.	Guinea pig serum + guinea pig placenta	Death in 3 min.
Apr. 18	231	250 gm.	0.25 c.c.	Guinea pig serum + guinea pig placenta	Mild symptoms for 10 min.; recovery.
Apr. 18	233	250 gm.	0.5 c.c.	Guinea pig serum + guinea pig placenta	Death in 5 min.

the minute particles of tissue remaining in suspension after centrifuging, then the fluid injected into guinea pigs 219 or 220 and that injected into 226 or 227 should be equally toxic, since in both cases the sera were digested with the same tissue. The same should be true about the toxicity of the portions of human and guinea pig sera, respectively, both of which were digested with guinea pig placenta. As can be seen from the results, however, whereas the toxicity of guinea pig serum increased during the incubation more than 800 per cent., the human serum, on the contrary, lost its natural toxicity for guinea pigs. The results of this experiment seem to show conclusively that the toxic substances in the Abderhalden test originate from the serum.

DIGESTED SERUM IS TOXIC ONLY FOR HOMOLOGOUS ANIMALS.

Although the results of the preceding experiment show that the toxicity of the end-products of the Abderhalden reaction originates

from the serum, the fact that the pregnant human serum, having been treated in a way absolutely similar to that of a pregnant guinea pig, failed to develop toxic properties suggests further study. There seem to be two possible explanations of this difference. Either the split products of human serum are in general less toxic than those of guinea pig serum, or the products of digestion of any serum are toxic to homologous animals only. In order to determine which is the case, the following experiments were undertaken.

On Apr. 21 5 c.c. of the serum of a pregnant rabbit and guinea pig, respectively, were put in test-tubes with 2 gm. of boiled human placenta tissue in each and left on ice over night. A similar set of two tubes was made up with human placenta and male instead of pregnant serum of a rabbit and a guinea pig. Early the next morning the contents of all four tubes were centrifuged, and after separation from the sediment the sera were transferred to the thermostat for sixteen hours. At the end of this time the contents of the tubes were injected intravenously into male guinea pigs and rabbits with the results indicated in table VI.

TABLE VI.

Date	Animal.	Animal No.	Weight.	Dose.	Material injected.	Results.
Apr. 22	Rabbit	101	1,075 gm.	2 c.c.	Pregnant rabbit serum	Acute symptoms. Found dead next morning.
Apr. 22	Rabbit	102	1,090 gm.	1 c.c.	Pregnant rabbit serum	Grave symptoms. Recovered.
Apr. 22	Rabbit	103	1,015 gm.	5 c.c.	Male rabbit serum	No symptoms.
Apr. 22	Rabbit	104	1,025 gm.	3 c.c.	Pregnant guinea pig serum	No symptoms.
Apr. 22	Rabbit	105	1,025 gm.	3 c.c.	Male guinea pig serum	Slight symptoms; recovered in 15 min.
Apr. 22	Guinea pig	306	255 gm.	1.5 c.c.	Pregnant rabbit serum	Restless; recovered in 5 min.
Apr. 22	Guinea pig	307	250 gm.	1.5 c.c.	Male rabbit serum	Coughing; recovered in 30 min.
Apr. 22	Guinea pig	308	255 gm.	0.5 c.c.	Pregnant guinea pig serum	Dead in 2 min.
Apr. 22	Guinea pig	309	245 gm.	4 c.c.	Male guinea pig serum	No symptoms.

It would seem, therefore, that the products of autodigestion of human serum must be toxic for human beings if they are toxic at all. In order to determine if this was the case, serum of a pregnant woman was treated in a way exactly similar to that just described for the serum of a rabbit or guinea pig. After separation from the placenta and subsequent incubation at 37° C., it was injected at in-

tervals during the process of autodigestion in the dose of 0.05 c.c. into the skin of several members of the laboratory staff. Parallel injections were made also with 0.05 c.c. of male serum treated in a similar manner. Whereas the injections of male serum did not cause any symptoms, the pregnant serum showed unmistakable signs of toxicity. Moreover, this toxicity seemed to be increasing for a certain time with the progress of digestion, since the injection made six or twelve hours after the beginning of autodigestion did not cause any symptoms. The injection of the material digested eighteen, twenty-four, and thirty hours was followed by a strong local reaction which resulted in the first two cases in a distinct reddened area with marked sensitiveness, and in the last case in the production of a sterile pustule, which appeared about ten hours after injection and remained for over twenty-four hours (14). The reactions at the site of the inoculation with the same serum later in the process of autodigestion showed marked diminution in toxicity of the serum, and after forty-eight hours the serum lost all its toxicity.<sup>2</sup>

TOXICITY IS CHARACTERISTIC ONLY OF A CERTAIN STAGE OF THE  
AUTODIGESTION OF THE SERUM.

In the experiments reported above it was assumed on the basis of previous results that toxicity of the serum, previously treated with kaolin or placenta protein, respectively, developed during its subsequent incubation at 37° C. is due to the process of autodigestion. If this is true, the loss of toxicity of such a serum, if incubation is prolonged, may be due to the further cleavage of the split products of the serum beyond the toxic stage. That both these assumptions are correct may be seen from the following experiments (table VII).

6 c.c. of pregnant guinea pig serum were placed in four test-tubes, 1.5 c.c. in each, and left on ice for sixteen hours in contact with 0.5 gm. of boiled human

<sup>2</sup> Similar findings were reported by Vaughan, Cumming, and Wright (15). According to these authors the serum or extracts of organs from actively anaphylactic guinea pigs after half an hour's contact with antigen furnished (intracardial injection) poisons which were acutely fatal, while sera or extracts of organs of normal animals did not. These authors report that after longer contact these poisons were destroyed.

placenta tissue in each tube. At the end of this time the contents of all tubes were thoroughly centrifuged, the serum was separated and transferred to four tubes containing 4.5 c.c. of physiological salt solution, 4.5 c.c. of normal guinea pig serum, 4.5 c.c. of solution of serum albumin, and 4.5 c.c. of a solution of serum globulin, respectively. All the tubes were placed in the incubator and at the end of sixteen hours the contents were injected intravenously into normal guinea pigs.

TABLE VII.

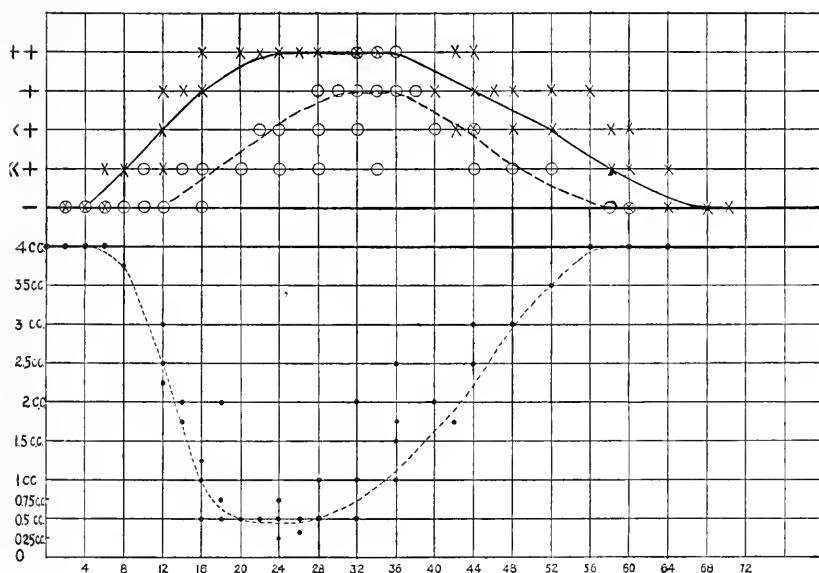
Date.	Animal No.	Weight.	Dose.	Material injected.	Results.
May 8	320	240 gm.	1 c.c.	Treated serum + salt solution	No symptoms.
May 8	321	250 gm.	1.5 c.c.	Treated serum + salt solution	Grave symptoms. Recovery.
May 8	322	250 gm.	2 c.c.	Treated serum + salt solution	Death in 2 min.
May 8	323	240 gm.	4 c.c.	Treated serum + normal serum	Very mild symptoms.
May 8	324	250 gm.	4 c.c.	Treated serum + serum albumin	Mild symptoms.
May 8	326	253 gm.	4 c.c.	Treated serum + serum globulin	Death in 2 min.
May 8	327	255 gm.	1.5 c.c.	Treated serum + serum globulin	Grave symptoms. Found dead next morning.

It is evident that the excess of normal serum or serum albumin, which, as I showed (16), stops the autodigestion of serum, as tested by the appearance of dialyzable ninhydrin reacting substances, prevents also the formation of toxic products in the serum. Serum globulin, which was shown to promote the autodigestion of serum, does not stop the formation of anaphylatoxin. That such toxic sera, during prolonged incubation, lose their toxicity due to further digestion of toxic compounds, can be seen from the following experiment.

On May 2 at 4 P.M. five pregnant guinea pigs were bled, and 40 c.c. of the serum were placed on ice in a tube with 20 gm. of boiled human placenta. At 8 A.M. on May 3, after sixteen hours, the serum was separated by centrifuging and transferred to the thermostat. In the progress of incubation samples of the serum were taken at intervals and tested simultaneously for toxicity upon normal guinea pigs as well as for dialyzable products of digestion. The latter test was done by transferring 1.5 c.c. of the serum from the incubator into a dialyzing thimble, as for the Abderhalden test (but without substratum), and placing the container with the thimble on ice. Under these circumstances, as I have shown, no further digestion takes place, and if there are any dialyzable products in the serum, they are not prevented from diffusing into the outer liquid in the container. Dialysis is allowed to proceed for twelve hours and at the end of this time the ninhydrin as well as biuret tests are made. The results of the experiment are shown in table VIII.

TABLE VIII.

Date.	Duration of incubation.	Weight.	Dose.	Results.	Biuret test.	Ninhydrin test.
May 3, 3 P.M.	6 hrs.	250 gm.	0.5 c.c.	No symptoms	-	-
May 3, 9 P.M.	12 hrs.	250 gm.	0.5 c.c.	Death in 10 min.	-	<+
May 4, 9 A.M.	24 hrs.	245 gm.	0.5 c.c.	Death in 5 min.	<+	++
May 4, 9 P.M.	36 hrs.	240 gm.	0.5 c.c.	Grave symptoms	<<+	++
				Recovery		
May 4, 9 P.M.	36 hrs.	245 gm.	1 c.c.	Death in 10 min.	<<+	+
May 5, 9 A.M.	48 hrs.	250 gm.	1 c.c.	No symptoms	-	<<+
May 5, 9 A.M.	48 hrs.	250 gm.	2 c.c.	Mild symptoms	-	<<+
May 5, 9 A.M.	60 hrs.	255 gm.	2 c.c.	No symptoms	-	-
May 6, 9 A.M.	72 hrs.	253 gm.	2 c.c.	No symptoms	-	-



TEXT-FIG. 1. The average changes of the serum, recorded in several successive experiments. The numbers placed on abscissæ represent the intervals of time at which the examinations were made. The ordinates show the intensity of the respective reactions. The lowest dotted curve represents the toxicity developed in the serum during incubation, the ordinates expressing the toxicity of serum. The two upper curves represent the intensity of the biuret (dotted line) and ninhydrin (solid line) tests and are expressed in arbitrary terms of negative, very weakly positive, weakly positive, positive, and strongly positive reactions.

The results, however, are not absolutely constant, since the rate of digestion may be influenced by the amount of antibody as well as by the antitryptic index, both of which vary in individual cases, and if both happen to exert their influence in the same direction the autodigestion may be markedly delayed or accelerated, as the case may be.

Comparison of these curves suggests definitely that the toxicity of the serum is in direct relation to its digestion, and that, if this digestion is allowed to proceed far enough, the cleavage products are broken down beyond the stage at which they possess the molecular configuration responsible both for the toxicity as well as for the typical color reactions (text-figure 1).

#### DISCUSSION.

Assuming that the anaphylactic syndrome, as it occurs in the body of a sensitized animal upon introduction of antigen, is due to the toxicity of the products of specific parenteral digestion of antigen, Friedberger attempted to produce the same substances *in vitro* by adding fresh complement to the antigen-antibody combination. The poisons obtained in this way, when injected in normal animals, were able to produce typical anaphylactic phenomena. Assuming that the substance obtained *in vitro* was identical with that formed *in vivo* in anaphylaxis, he called this poison anaphylatoxin. However, in the later studies of Friedberger, and especially in the work of other authors, the term anaphylatoxin was applied to any poisonous substance which was able to produce in normal animals the phenomena similar to those of anaphylactic syndrome.

Thus various authors have called anaphylatoxin the chemical poisons obtained by Vaughan from bacteria; and Nathan, Bordet, and Mutermilch called anaphylatoxins substances obtained by them from the serum by adsorption with inert substances. The attempts of these authors to explain the nature of anaphylatoxin seem to be generally inadequate, because even though, as my experiments also have confirmed, by digestion of serum with kaolin, for instance, one can produce from serum a substance which is similar in its physiological action, on the one hand, to the anaphylatoxin of Friedberger, and, on the other hand, to the chemical poison of Vaughan, its identity with one or the other is not proved by this similarity alone.

Inasmuch as my experiments show that the changes which the serum undergoes under the influence of kaolin are identical with the changes taking place in the immune serum as a result of its interaction with the antigen, they seem to throw light on the nature of anaphylatoxin. Since the process of the formation of toxic products in both cases is established as identical, the similarity of the biological properties of the respective end-products may speak for their identity. These findings, taken in connection with the results reported by me in the previous communication, suggest that the nature of anaphylatoxin is as follows: Fresh serum<sup>3</sup> contains normal proteolytic ferments whose digestive action *in vivo* is inhibited by the simultaneous presence of some antitryptic elements. This antitrypsin can be removed from the serum *in vitro* by two independent processes: one non-specific, a simple mechanical adsorption by means of excess of some organic as well as some inorganic substances; the other specific, an inactivation of the antitryptic properties of the serum taking place as a result of the physicochemical changes in the serum induced by the specific interaction between the antigen and the antibody of the immune serum. The removal of the inhibiting antitryptic action of the serum by either method is followed by the restitution of the activity of the normal proteolytic enzyme, which digests the globulin of the serum.<sup>4</sup> At a certain stage of this autodigestion the serum exhibits toxic properties which are able to cause a typical reaction (local or general) in homologous animals.

#### SUMMARY.

I. The union of fresh serum of pregnant or immunized animals with the corresponding boiled protein (substratum) is accompanied by the formation of poisonous substances.

<sup>3</sup> Heated serum is free from these ferments unless reactivated by the addition of fresh serum (complement).

<sup>4</sup> It is probable that such proteolytic ferments, activated (by the removal of the inhibition exerted by serum antitrypsin) through the combination of antigen and antibody *in vivo*, may attack also the sensitized circulating antigen. What is important, however, in relation to the recent theories of immunity advanced by Abderhalden, is the fact that such ferments are not specific and when placed in contact with the coagulated antigen *in vitro* are absolutely unable to digest the latter.

2. The poison originates from the serum as a result of its autodigestion, and not from the substratum.

3. The process of autodigestion may be determined by the specific or non-specific removal of the antitrypsin of the serum.

4. The poisons originating from the serum are toxic only for homologous animals.

5. The autodigestion of the serum, if allowed to proceed far enough, may go beyond the toxic stage.

6. The biological properties of these poisons indicate their close similarity to the anaphylatoxin, and suggest that the anaphylatoxin of Friedberger is a product of the autodigestion of serum, and not of the protein outside of the serum.

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# THE WIDE-SPREAD DISTRIBUTION OF DIPHTHEROIDS AND THEIR OCCURRENCE IN VARIOUS LESIONS OF HUMAN TISSUES.\*

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## PLATE 37.

The term diphtheroid is here used with the meaning employed by writers in recent literature, particularly as regards the description of organisms of supposed etiological significance. It is not our intention to classify the diphtheroids as a separate species. Many writers, however, suggest an intimate relation or even identity between diphtheroids and certain streptothrices, which may exist in some instances. The term pseudodiphtheria as applied to *Bacillus xerosis* or *Bacillus hoffmanni* would seem to represent a synonym, except that by a diphtheroid an organism outside of the diphtheria group is apparently meant. By a diphtheroid is indicated, therefore, a non-acid-fast, Gram-positive bacillus resembling morphologically, at least in one or more of its forms, the bacillus of diphtheria. The diphtheroids may present either bipolar granules, barred arrangement of the chromatin, or, as is usually the case, shapes corresponding to involution types of *Bacillus diphtheriæ*; *i. e.*, club, retort, mandolin, or other forms of unusual morphology.

In the problems of the etiology of such diseases as leprosy, Hodgkin's disease, and general paresis, there have been described organisms which are diphtheroidal in morphology and to which causal properties have been ascribed in whole or in part. In certain instances these organisms have fallen almost into oblivion since the true causal agents have been discovered, postulated, and accepted; in others they are held partly in abeyance; while in still others certain diphtheroidal bacilli are supported, although not finally accepted.

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There seems to be a tendency to view these diphtheroids, when found in the course of etiological investigation, from the standpoint of causal relation rather than from the more probable aspect of contamination. This is especially true of those organisms cultivated from tissues which are sterile as far as accidental contamination is concerned. Many strains grow but feebly, requiring special food-stuffs, while others cannot as yet be made to grow away from the original tissue, even under the most favorable conditions. It is a common practice to regard slow, delicate, or fastidious growth as indicative of pathogenicity, and to be incompatible with contaminating saprophytic properties. Therefore, it would seem that the widespread distribution of this type of organism in the human host and in nature as a whole is not fully realized. When the prevalent *Bacillus subtilis*, *Bacillus proteus*, staphylococcus, or other ordinary aerobic organisms are met with in bacteriologic work, they are discarded as irrelevant contaminants. However, should a diphtheroid be chanced upon, particularly if it be somewhat difficult to cultivate, one is apt to assume an etiological significance for this particular organism, and to undertake the establishment of such a property, without sufficiently considering the likelihood of its being present as an accidental or associated non-pathogen. Many diphtheroidal organisms of the acid-fast group, such as *Bacillus smegmatis*, are extremely difficult of cultivation, yet as far as is known they are non-pathogenic. The more rapidly and luxuriantly growing types of the group of diphtheroids are not easily mistaken, since they are so frequently encountered upon the skin surface, in the hair, and particularly in the protected natural cavities, the conjunctiva and genitalia. Even here, however, occasionally other types are encountered, which being apparently less saprophytic attract more attention.

In many instances organisms are obtained from fresh sterile human tissues which invite serious consideration as the causal factors of pathologic conditions on account of their source, their scarcity, their slow and often fastidious growth, and their diphtheroidal morphology. It need only be emphasized here that numerous workers have cultivated organisms from normal tissues. Wolbach and Saiki (1), after anesthetizing healthy dogs and searing the abdominal

walls with hot irons and employing instruments thoroughly flamed, succeeded in cultivating from the liver of these animals in twenty-one out of twenty-three instances an anaerobic spore-bearing bacillus. Nicholls (2) and Ford (3) have also shown that bacteria can be recovered from healthy organs by cultivation. Wrosczek (4) has demonstrated that bacteria can pass from the alimentary tract by feeding cultures of non-pathogenic, pigment-producing organisms to animals and recovering these from various organs.

The various conditions in which members of this group of organisms have been isolated and considered as causal factors embrace a large number of diseases. We wish briefly to discuss the more important of these conditions.

With the exception of the study of the diphtheroids of leprosy, there has been no attempt to confirm the findings of the different workers upon the various diseases in which such organisms have been described. Our purpose is to indicate the broad latitude of this large group, the scope of which seems to have been only partially realized in the past.

*The Eye.*—Probably one of the first conditions to be ascribed to a diphtheroid was what is known as xerosis conjunctivæ. From this condition an organism was repeatedly cultivated which was designated *Bacillus xerosis* by Kuschbert (5) and Neisser in 1884. Its presence, however, was later shown to be incidental and not connected with the production of the condition. Under the name of *Bacillus xerosis*, as pointed out by Park and Williams (6), observers have described organisms of widely varying characteristics, which serves to illustrate the general confusion concerning the majority of these organisms of the diphtheroid group as a whole.

*Brain and Cerebrospinal Fluids.*—The repeated recovery of an organism of a diphtheroid nature from the brains of general paretics led Robertson (7), Hoag (8), Robertson (9), McRae and Jeffry, and others to regard it as probable that this organism was the pathogenic factor, although no experiment furnished sufficient weight for the final acceptance of this hypothesis. Orr, Rows, and Robertson (10) considered that they had substantiated *Bacillus paralyticus* of Robertson by the experimental histopathological lesions produced in the rabbit. Gay and Southard (11), in the examination of cerebro-

spinal fluids removed post mortem from the brains of fourteen cases of general paresis, failed to obtain a diphtheroid in any instance. The demonstration of *Treponema pallidum* in the brains of such conditions by Noguchi and Moore (12), Noguchi (13), and by Nichols and Hough (14) has banished all thought of this factor. Here again we have a striking example of the mistaken impressions received concerning the part of the diphtheroids isolated from tissues. In this connection it should be noted that in the routine study of cerebrospinal fluids, diphtheroidal organisms are sometimes encountered, the significance of which seems to vary in different cases. To illustrate this fact we shall cite a few typical instances. One fluid which when freshly drawn was reported negative for bacteria was found to contain after two days' standing numerous thin, beaded, sharp-ended, Gram-positive diphtheroids, easily grown in pure culture. A second case, that of a four day infant, showed at autopsy a general purulent meningitis from extension of a *Staphylococcus aureus* infection of an ulcerated spina bifida sac. Twenty-four hour cultures made from the spinal meninges showed numerous staphylococci with a few colonies of a Gram-positive diphtheroid. In both cases the organisms were evidently contaminators. It is more difficult, however, to account for the organism found in a third case. This was an adult male with high temperature and opisthotonus. On lumbar puncture forty cubic centimeters of a slightly turbid fluid were removed, which upon immediate centrifugalization showed many pus cells and numerous large, irregular, Gram-positive bacilli, varying from solid coccoid forms to those simulating the large, barred, club forms of *Bacillus xerosis*. These were found to be still numerous in a specimen taken twenty-four hours later, but subsequently they diminished in number until they disappeared, and the case recovered about a week after the first puncture. Upon none of the ordinary media, aerobically or anaerobically, could more than a slight, temporary multiplication be obtained.

*Blood Cultures.*—In the course of routine blood culture work, diphtheroids occasionally have grown out on the medium employed either together with organisms such as *Bacillus typhosus* and *Streptococcus pyogenes* or in pure culture. Their occurrence in conjunction with known pathogens, together with the fact that refinement

of technique diminishes the frequency of their occurrence, stamps them as frank contaminators. Their isolation, therefore, in blood culture from diseases of unknown etiology, such as typhus fever (15), etc., must be regarded with due conservatism. The recovery of non-pathogenic, non-toxin-producing strains of supposed *Bacillus diphtheriae* in cases considered to be diphtheria septicemia, in certain instances may be thus explained. That there may occasionally occur more or less generalized invasion of diphtheroids, however, is held probable. An instance suggesting this was encountered in a case of systemic blastomycosis which came to autopsy. In the purulent material from abscesses in the neck and pancreas and from cavities in the lungs which contained numerous yeast cells there were demonstrated in smears many Gram-positive diphtheroids. Cultures were obtained from these lesions which proved to be identical.

An interesting case that might be mentioned here was one in which pus was obtained upon aspiration of the chest, that showed in stained smears very numerous thin, non-acid-fast diphtheroids, easily grown in pure culture. The case subsequently recovered and was lost sight of.

*Lymphoid Tissues.*—During the past summer lymph nodes, usually inguinal or femoral, have been aspirated in approximately thirty cases presenting themselves at the Charity Hospital, in order to determine the possibility of *Bacillus pestis* infection. In several of these cases a few diphtheroids have been found in the smears obtained by puncture which with a simple stain were bipolar. As has been pointed out by one of us (16), it is important to bear this fact in mind in the study of simple stained smear preparations for the diagnosis of early plague infection. From three of these lymph node aspirations cultures of these confounding organisms have been recovered. It is worthy of note that their presence seems much more frequent in chronic hyperplasias than in acute infections of lymph nodes.

The lymphadenopathy which constitutes the lesion known as Hodgkin's disease has but recently been ascribed to a diphtheroidal type of organism. From the lesions Fraenkel and Much (17), de Negri and Mieremet (18), Bunting and Yates (19), Billings and Rosenow (20), Lanford (21), and others have reported the recovery

of diphtheroids of pigment- and non-pigment-producing varieties. Lanford reports the recovery of two strains of these bacilli from the spleen of splenic anemia. Bunting, Yates, and Kristjanson (22) have recently reported the cultivation of their *Bacillus hodgkini* from the spleen of so called splenic anemia. They argue from these findings a relation between this condition and Hodgkin's disease. We have cultivated diphtheroidal organisms from tuberculous glands, lymphosarcoma, and Hodgkin's disease, but have not attempted to carry out a comparative study of our cultures and those isolated from Hodgkin's disease by other workers. Strains of diphtheroids resembling those recovered from lesions of Hodgkin's disease have been cultivated by various workers from splenic anemia, lymphosarcoma, tuberculous adenitis, and other lesions. While lesions have been produced locally by the injection of these organisms, no general picture of the disease has resulted. Still it must be remembered that generalized lesions have been produced with known saprophytes intravenously injected in large amounts and at sufficiently frequent intervals. Again, in studies bearing upon the causation of hepatic cirrhosis de Vieche (23) by introducing certain bacteria into the intestinal tract, and Weaver (24) by subcutaneous injection of bacilli of the colon group obtained definite proliferation of the interstitial connective tissue of the liver. In this connection Hektoen's work (25) is of special interest, as he produced similar lesions with a pseudodiphtheria or diphtheroidal organism. Furthermore, we can here refer to the acid-fast group which forms a more striking analogue for confusion in the identification of the Hansen bacillus culture. Duval and Harris (26) and Machow (27) have produced definite generalized nodular lesions in rabbits and white rats with intravenous injection of such saprophytes as the bacillus of timothy hay, *Bacillus smegmatis*, and the dung bacillus of Moeller.

*Tumors.*—In order to ascertain whether diphtheroids were present in tumors, bits of tissue from six specimens, usually warm from the operating room, were planted on various media. These specimens included excised nodules of two cases of lymphosarcoma, two leiomyomata, one from the uterus and the other from the back, a fibroma removed from the pectoral muscle, and a necrotic area of an hepatic carcinoma obtained post mortem. In all cases the surface

of the tissue was thoroughly seared and the material for cultivation removed from the deeper levels. After periods ranging from three days to two weeks colonies of diphtheroids of different types appeared on or about certain of the planted bits from all these except the uterine leiomyoma. In three cases diphtheroids alone were cultivated; while from the tumor of the back the staphylococcus, and from the liver the colon bacillus were also obtained. In several instances the formation of the colony within the tissue fragment, its enlargement, and final eruption upon the surface were watched, thus eliminating the possibility of contamination through faulty technique.

In extension of this work several cultures of more or less rapidly growing diphtheroids were obtained from other tissues removed at surgical operation and planted upon nutrient media. One of these organisms is of particular interest since it belongs to the acid-fast group. From an unopened appendix, supposedly sterile, a bit of the meso-appendix was removed and planted upon blood serum. After two weeks there appeared upon the tissue itself a cadmium yellow colony. Smear preparations when stained showed a typical Gram-positive diphtheroid, which when stained by Gabbett's method proved to be strongly acid-fast. Subplants grew well upon the ordinary laboratory media and liquefied completely blood serum and gelatin. The culture has not been definitely identified, but is apparently one of the many saprophytic acid-fast strains. This isolation presents an instance of the liability of occasional contamination with a strongly acid-resisting saprophyte in cultivation experiments.

*Leprosy.*—Kedrowski (28), Bayon (29), Williams (30), Wolbach and Honeij (31), and others have isolated and cultivated from the leprosy lesion diphtheroids to which they have assigned definite causal properties, each regarding his culture as a stage in the life cycle of the Hansen bacillus. Kedrowski (32), in particular, reasons a mutation of the non-acid-fast diphtheroid cultivated by him from the tissue of lepra nodules, because upon injection into animals, he recovered only an acid-fast growth. Campana (33) and Babes (34), who separated non-acid-fast diphtheroids and streptothricial forms supposedly in pure cultures from leprous tissues, consider that Kedrowski was working originally with a contaminated culture. Throughout this experimental work upon the cultivation of *Bacillus*

*lepra*, conducted by Duval and his assistants (35, 36), we have from time to time isolated similar non-acid-fast diphtheroids which when injected into animals have never shown evidence of having acquired acid-fast properties. These strains of diphtheroids have been maintained unchanged in culture for several years.

This confusion of acid-fast and non-acid-fast organisms recovered from the lesions of leprosy has a parallel in the following study of tuberculous tissues. For example, we have repeatedly recovered cultures of non-acid-fast diphtheroids from tissues, which by sections and smears have proved to be tuberculous. Several tissue bits were removed from a tuberculous axillary node. One of these was macerated and injected into a guinea pig that died of tuberculosis in five weeks. The remaining tissue fragments were planted in six tubes of various culture media and after incubating for one week, five of these showed staphylococci, either alone or mixed with diphtheroidal bacilli. In one tube the tissue appeared to have remained sterile. Smears from this, however, showed numerous small groups of diphtheroids, which were apparently in pure culture and subsequently became a comparatively free grower. The original tissue culture was later inoculated into a guinea pig, and after six weeks the animal showed signs of tuberculosis. From the lesions *Bacillus tuberculosis* was cultivated, but no non-acid-fast diphtheroid. In such an instance the contaminating diphtheroid and the specific acid-fast bacillus were known entities, and one cannot reason that the non-acid-fast diphtheroid is part of the life cycle of *Bacillus tuberculosis*. That Kedrowski (32) was handling a mixture of an acid-fast bacillus and a non-acid-fast diphtheroid, injection of which mixture into an animal occasioned the disappearance of the non-acid-fast diphtheroid and the recovery of the acid-fast organism, is suggested by this experiment.

For the purpose of ascertaining the liability of skin contamination in the leper, we obtained material from the Louisiana Leper Home. Nodules of tissue in the tubercular form were removed from areas which were previously sterilized with soap and water and tincture of iodine. Portions were planted upon agar, Loeffler's blood serum, and numerous special media. Surface cultures were made from the lesions of trophic types of the disease, as represented by cases with



contractures and atrophies and the partly or completely cicatrized areas of previous lesions. Similar cultures were made from macular lesions. As controls and to ascertain the prevalence of this type of organism upon the skin of lepers, similar cultures were made from uninvolved skin areas of eleven cases of various types of leprosy. Growths appeared in from one to ten days, and in many instances showed staphylococci and other contaminating organisms. In some the diphtheroids were so overgrown that they could not be recovered. The results are shown in table I.

TABLE I.

*Leprosy.*

No. and types of cases.	Media.	Results.
15 tissue bits, from 5 nodules excised from 4 patients with tubercular type	Tryptophane agar, tryptophane blood agar, tryptophane ascitic agar, and ordinary blood serum and nutrient agar	7 growths of diphtheroids from 4 cases. 3 heavy growers, one being dirty yellow, another dull red, and the third white. 4 slow growers, growth simulating that of <i>Streptococcus pyogenes</i> . All were non-acid-resisting.
5 plants from scrapings over nodules of 5 other cases with tubercular type	Blood serum and nutrient agar	2 growths of free growing diphtheroids. Non-acid-resisting.
10 plants from scrapings of macular lesions of 10 such cases	Blood serum and nutrient agar	No diphtheroids found.
12 plants from scrapings of lesions of 12 cases with trophic type	Blood serum and nutrient agar	9 growths of diphtheroids, 8 free growing, of which 4 were cream color and 4 white, 1 slow grower, streptococcus-like. 2 of these cultures showed streptothrices.
2 plants from conjunctival sac and 1 from a comedo	Blood serum and nutrient agar	All showed diphtheroids, those from the eye corresponding to <i>B. xerosis</i> .
11 plants from normal skin areas of 11 patients with various types	Blood serum and nutrient agar	6 growths of diphtheroids. Three were creamy, two lemon yellow, and one slow growing and streptococcus-like. 2 of these cultures showed streptothrices.

*Summary.*—From 45 different lepers presenting various forms of the disease, 27 growths of chromogenic, non-chromogenic, free growing, and slow growing diphtheroids were obtained. Six of these were from normal skin areas. It is noteworthy that none of these

organisms were acid-resisting when the ordinary standard methods of decolorization were employed.

All the various morphological characteristics of *Bacillus diphtheriae* were represented in these cultures. Many of them were shown to be weakly acid-resisting, if the mineral acids used to decolorize were as dilute as 2.0 per cent.

These results indicate the prevalence of bacilli of the diphtheroid type and the possibility of encountering them where the cultivation of *Bacillus leprae* is attempted.

*Skin*.—To determine the presence of diphtheroids in miscellaneous skin lesions, cases of pellagra, acne, marked comedones, etc., were selected. The skin areas were carefully cleansed with 95 per cent. alcohol, and the pus, parts of the scales, and the comedones were cultured on ordinary laboratory foodstuffs. Forty such plants were made, and while a large number contained staphylococci, twenty-five showed definite diphtheroidal bacilli, many of which were isolated in pure culture. Twenty-three were of a rapidly growing character, and two grew slowly and scantily. The majority of these diphtheroids presented a moist cream colored growth; one was salmon in color, one yellow, and some were quite white. All stained by Gram's method, and the salmon colored chromogenic strain was acid-resisting. This particular organism was recovered from a moist eczematous lesion of the neck. In two of the twenty-five tubes containing diphtheroids, unidentified streptothrices were found.

Various workers such as Castellani (37), Chalmers and O'Farrall (38), Chalmers and Stirling (39), and Johns (40) have cultivated from the hairs of trichonocardias a bacillus which they regard as an integral unit of the life cycle of the *Nocardia tenuis*; they also procured colonies of cocci. The bacillus from description and plates is of a distinctly diphtheroidal character. While, of course, we recognize that this type of organism might form a phase of higher plant life, still because of the presence of known irrelevant organisms in some of their cultures and in the light of the extensive presence of diphtheroids on the skin, the question of contamination is here again to be considered. We may mention particularly a case regarded by the dermatologist as probable sporotrichosis, which recovered rapidly with antisyphilitic treatment. Three nodular lesions were present,

one on the dorsum of the wrist and two on the forearm. After careful sterilization the skin was incised and several subcutaneous tissue bits were removed. These were planted in many tubes of various media, and after incubation for six days colonies appeared on the tissue bits in two of the tubes. Both were chromogenic. One was yellow, grew well in subplant, and presented chiefly short chubby bacilli, while the other was of a light yellow color and grew very feebly, showing marked pleomorphism.

The great liability of skin contamination by diphtheroids is again demonstrated by their cultivation from the various cutaneous disturbances selected for cultural study.

*Air.*—A series of eighteen plates measuring fourteen centimeters in diameter was exposed to the air for varying periods of time. Nine of these contained blood agar, and six contained blood serum. As an occasional liquefier destroys a plate of blood serum, blood agar was found to be preferable. Thirty minutes' exposure yielded a colony seeding which could be advantageously picked after the necessary incubation. Three different plates were exposed on different days so that the daily air currents would present different atmospheres for examination; likewise different rooms were chosen to form a varied environment of search. The plates were incubated for forty-eight hours, with results which are shown in table II.

TABLE II.  
*Aerobic Organisms.*

Plates.	Time.	Results.
4 plates, 14 cm. (2 of blood agar and 2 of blood serum)	15 min.	3 colonies of diphtheroids.
10 plates, 14 cm. (8 of blood agar and 2 of blood serum)	30 min.	29 colonies of diphtheroids. 7 colonies distinctly acid-fast.
4 plates, 14 cm. (2 of blood agar and 2 of blood serum)	45 min.	6 colonies of diphtheroids.

*Summary.*—18 plates showed 45 colonies of diphtheroids. Of these, 7 were of an acid-fast nature. They were mostly chromogenic and varied as follows: 17 salmon, 7 chocolate brown (probably in part due to the pigment of the blood agar), 6 lemon yellow, 5 pink, 4 white, 3 distinctly red, and 3 were colorless and grew slowly.

The colonies varied in size from 0.5 mm. to 4 mm., averaging about 2.5 mm. They were round and were sharply defined in contour. All were moist with the

exception of the three small, colorless colonies which grew slowly in transplants and assumed the appearance of an influenza culture.

The results demonstrate that diphtheroidal bacilli are readily obtained from the atmosphere, and show the possibility of contamination through this means.

Aerobic diphtheroids have been recovered by McNaught (41) from water. We have also isolated them from urine and feces.

#### DISCUSSION AND SUMMARY.

It is evident from this work and from the work of others that organisms having diphtheroidal morphology are wide-spread in their distribution in the human body. They are readily isolated from miscellaneous skin lesions, probably thriving better in pathological soils as saprophytes than in normal skin, although they can be obtained quite easily from the latter. Various strains can also be cultivated from the air, at least in this environment.

Aside from the air and superficial parts of the human body, diphtheroids have frequently been isolated from the deeper regions. Hoag, working on general paresis and certain conditions of the lung, cultivated 199 diphtheroids, the majority of which were from the lungs and respiratory tract, also in some instances from the liver and spleen. He considered 146 of these isolations to be identical and classified them as organism x. Robertson, McRae, and Jeffry likewise in cases of dementia paralytica isolated what they considered an identical strain which was closely related to the Klebs-Loeffler bacillus, from the inflamed ileum, stomach, tonsils or pharynx, bronchi, lung, and brain. They differentiated their organism from *Bacillus hoffmanni* and *Bacillus xerosis*.

The fact that these organisms have a somewhat unusual morphology, resembling as they do an organism of known pathogenicity, namely, *Bacillus diphtheriae*, should form no basis for assuming that they, too, are apt to possess these qualities. When we consider the number of simple bacillary contaminants analogous to such organisms as *Bacillus anthracis*, *Bacillus typhosus*, etc., we can readily see how frequently this occurs.

The majority of the diphtheroids isolated by us stain by Gram's

method; nearly all fail to resist the standard acid-fast methods, although use of the very low strengths of mineral acids allows the fixed stain to remain in whole or in part. There is consequently nothing unique in their tinctorial properties.

These bacillary strains differ considerably in their powers of cultivation. While the majority grow readily upon the ordinary laboratory foodstuffs, others require special products for their growth and still others cannot be grown even where special effort has been made. The fact that many strains grow slowly no doubt enhances their consideration as disease producers, as it seems to argue their difficult adaptation from their preëxisting site to an artificial environment. This reasoning does not apply when we consider the numerous causal agents that can so readily be cultivated directly from the diseased tissue, and again the fact that many saprophytes in their natural habitat are incapable of growth *in vitro* (figure 1).

The most striking of the biological features of these cultures is their wide range of pigment production. While many are white or creamy white, the majority range from a canary yellow to a distinct crimson, and one of the cultures recovered from a leper was a very deep dusky red or garnet. Numerous combined shades of yellow and pink, including such colors as salmon and orange, were present. Some cultures were distinctly pink, others varied in depth of color to a crimson. While many cultures of identical colors were obtained, the majority of strains showed some slight variance of shade.

While, of course, the inoculation of diphtheroidal cultures produces varying degrees of localized inflammation if employed in sufficient dosage, probably for the two-fold reason of acting as a foreign body and liberating proteolytic substances, still the diphtheroids isolated in the past have never been able to reproduce the clinical picture of the various diseases from which they were cultivated. The apparent reproduction of lesions in general paresis, in hepatic cirrhosis, and in leprosy does not form an ultimate postulate for the acceptance of organisms as specific etiological entities. This is evidenced by the fact that certain known saprophytic organisms when used as controls are capable of producing the same results.

It seems clear that organisms as broadly distributed as the diph-

theroids should be suspected as contaminators when met in experiments bearing upon etiology. They should be viewed primarily in the same light as the staphylococcus and the various known contaminating bacteria. It is apparent that this type of organism has been regarded too much from the standpoint of pathogenicity. It is, of course, probable that among this large group there may be a few pathogenic types.

#### CONCLUSIONS.

1. Diphtheroids are widely distributed in nature. They are present in the air, on the body surface, and at times through contamination or are indigenous in the deeper tissues.

2. Diphtheroids constitute a broader field of saprophytism than is generally appreciated. While some strains may represent pathogens, their aggregate is patently not of the disease-producing variety.

3. Diphtheroids can be cultivated from various pathological tissues to which they bear no etiological relation, such as lesions of tuberculosis, leprosy, blastomycosis, tertiary syphilis, and tumors of various types.

We wish to extend our thanks to Dr. C. W. Duval for suggestions, and to Dr. A. Mattes and Miss Alice B. Moerner for assistance in certain features of this work. We wish also to thank Dr. Ralph Hopkins, Visiting Physician in charge of the Louisiana Leper Home, who made it possible to obtain certain material.

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#### EXPLANATION OF PLATE 37.

The illustration affords some idea of the growth and appearance of representative isolations in the chrome series.

Cultures D 17, D 29, and D 32 are from tumors, and D 21 is from an appendix removed at operation. Cultures D 74, D 75, D 76, D 79, D 81, D 85, and D 88 are chromes recovered from air. D 58 is from the feces of a case dead of B.

*acrogenes capsulatus* infection. D 52 was obtained from a blood culture. D 49 is one of the chromogenic strains cultivated from acne.

D 17, D 21, D 29, and D 52 present irregular, granular, wrinkled growths, somewhat dry in appearance. Culture D 21 imbeds itself slightly in blood serum, possessing to a minor extent a liquefying property.

D 49, D 58, D 79, D 81, and D 85 show a more or less smooth surface and are moist.

D 32, D 74, D 75, D 76, and D 88 grow very heavily, appearing quite thick; the surfaces are smooth and glistening, and the growths are extremely moist.

The chromes produced by these cultures are as follows: 17, a cream yellow; 21, pink; 29, deep orange; 32, red; 49, pink, but darker than 21, and its growth is smooth; 52, cream white or ivory; 58, a very dark pink approaching red; 74, salmon; 75, cadmium; 76, orange; 79, canary yellow; 81, cream; 85, yellow; 88, a pinkish salmon.



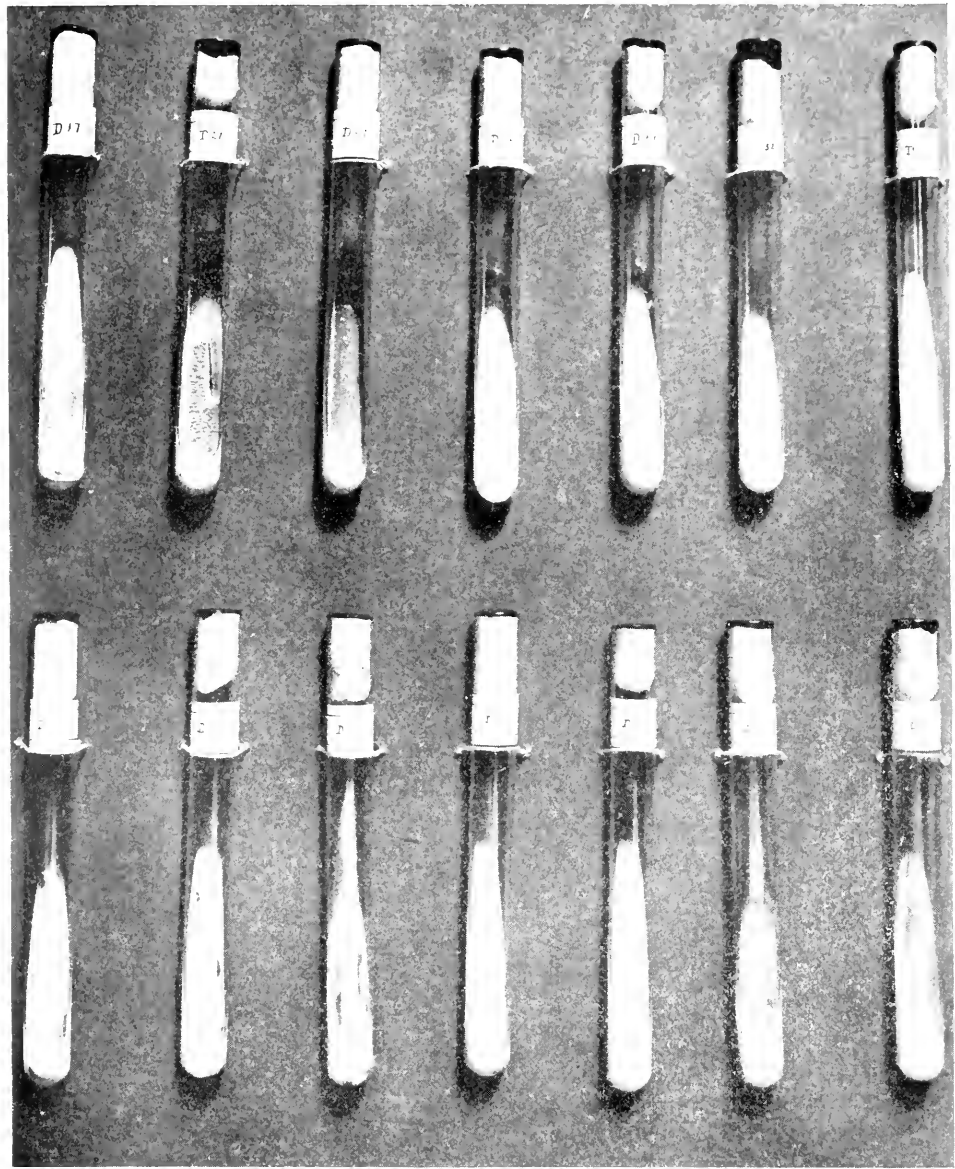


FIG. 1.

(Harris and Wade: Distribution of Diptheroids.)



## DIFFUSION AND SURVIVAL OF THE POLIOMYELITIC VIRUS.\*

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(From the Laboratories of The Rockefeller Institute for Medical Research.)

In earlier publications<sup>1</sup> we have presented data bearing on the view that the minute microörganism cultivated from poliomyelitic tissues and described by Flexner and Noguchi,<sup>2</sup> constitutes the cause of epidemic poliomyelitis. In this paper we propose to deal with the power of diffusion and the period of survival of the virus of poliomyelitis contained in the central nervous organs, since these factors affect the evidence submitted concerning the specific activities of the microörganism.

We have alluded in a previous paper to the factor of the survival of the virus at thermostatic temperature,<sup>3</sup> but this has never been accurately determined and could only be inferred from experiments made to determine other qualities of the virus. The question of the power of diffusion of the virus from a fragment of infected nervous tissue into the surrounding medium has never been directly considered.

The capacity for diffusion and survival of the virus bears directly upon the evidences that have been brought forward to support the specific nature of the microörganism under consideration. We now know that certain cultural strains after a number of removals from the nervous tissues from which they were derived, and great dilution, may still set up poliomyelitis in monkeys upon inoculation. Although the experiments demonstrating this fact are convincing, it is nevertheless desirable to ascertain by direct experiment what the capacity for diffusion and survival of the ordinary virus of

\* Received for publication, March 1, 1915.

<sup>1</sup> Flexner, S., and Noguchi, H., *Jour. Exper. Med.*, 1913, xviii, 461. Flexner, S., Noguchi, H., and Amoss, H. L., *ibid.*, 1915, xxi, 91.

<sup>2</sup> Flexner and Noguchi, *loc. cit.*

<sup>3</sup> Flexner, Noguchi, and Amoss, *loc. cit.*

poliomyelitis is, in order to exclude the remote possibility of the persistence within the cultures of an active agent of invisible character.

#### EXPERIMENTAL.

The method employed to study the diffusion and survival of the virus is simple. Fragments of brain tissue containing the virus are placed in tubes of ascitic fluid and kidney fragment overlaid with paraffin oil, the medium employed for actual cultivation tests. The tubes are not enclosed in anaerobic jars, and are kept at 37° C. From time to time a tube is removed from the thermostat, and a quantity of the ascitic fluid in proximity to the brain tissue is pipetted off, after which the brain fragment is removed. Both fluid and fragment are used for inoculating monkeys by intracerebral injection. Since the tubes have not been enclosed in an atmosphere of hydrogen, multiplication of the minute organism will probably not take place. Whether or not it does take place can be determined by suitable tests.

It is essential to employ a strain of the virus of high virulence. This was done in the experiments by selecting a monkey from which to take the brain fragments, which had succumbed to an inoculation of a highly active sample of the ordinary virus of poliomyelitis. The virus is capable of producing paralysis when injected intracerebrally in quantities of 0.1 to 0.3 of a cubic centimeter of a Berkefeld filtrate of a 5 per cent. emulsion of the spinal cord. Before the brain tissue submitted to incubation is employed for inoculation, an emulsion is prepared containing 0.2 of a gram of the tissue which inoculated intracerebrally into a control animal causes paralysis in a period varying from five to eight days. Subsequently it is necessary merely to remove the inoculated tissue at stated intervals from the thermostat and to inoculate the corresponding quantity of 0.2 of a gram of brain fragment, in order to determine the period during which the virus survives in an active state under the conditions of the experiment.

The tests on the diffusion of the virus were conducted with quantities of 0.2 of a cubic centimeter of the ascitic fluid adjacent to the brain fragment which had been pipetted off at intervals after

incubation. The quantity of 0.2 of a cubic centimeter was chosen because it represents the average quantity of the fluid medium which is transferred from the original to subsequent tubes of ascitic fluid medium in the course of the cultivation of the minute micro-organism from infected brain tissue. Should the virus diffuse freely from the brain tissue into the surrounding medium, the ascitic fluid should then become infectious. No experiments have ever been conducted to ascertain whether diffusion takes place at all, or the extent to which it does occur. Should the diffusion be so slight that 0.2 of a cubic centimeter of the ascitic fluid contains less than a minimal dose capable of producing poliomyelitis in the monkey, the transfer of this quantity of the fluid medium from one tube to another could not result in the carrying over of an effective amount of the virus.

#### INOCULATION OF BRAIN TISSUE.

*Experiment 1.*—Control. Jan. 7. Injected intracerebrally into a *Macacus rhesus* an emulsion containing 0.2 gm. of brain removed from a recently paralyzed monkey. Jan. 10. Excitable. Jan. 11, A.M. Tremor, ataxia. P.M. Arms and legs weak; later prostrate. Jan. 12. Died. Typical poliomyelitis.

*Experiment 2.*—Brain tissue incubated for 10 days. Jan. 18. Injected intracerebrally into a *Macacus rhesus* 0.2 gm. of brain tissue, in emulsion, derived from the same animal as in experiment 1, which had been incubated in ascitic fluid kidney medium for 10 days. Jan. 31, A.M. Arms paralyzed, paralysis of right side of face. P.M. Prostrate. Feb. 1. Etherized. Typical poliomyelitis.

*Experiment 3.*—Brain tissue incubated for 20 days. Jan. 28. Same procedure as in experiment 2. Feb. 4. Excitable, ataxic, left arm paralyzed. Feb. 6. Prostrate. Feb. 8. Died. Typical poliomyelitis.

*Experiment 4.*—Brain tissue incubated for 30 days. Feb. 9. Same procedure as in experiment 2. No effects produced.

*Experiment 5.*—Brain tissue incubated for 40 days. Mar. 1. Same procedure as in experiment 2. No effects produced.

#### INOCULATION OF ASCITIC FLUID.

*Experiment 6.*—Ascitic fluid incubated for 10 days. Jan. 18. 0.2 c.c. of ascitic fluid adjacent to brain tissue employed in experiment 2, removed by pipette after 10 days' incubation, inoculated intracerebrally into a *Macacus rhesus*. No effects produced.

*Experiment 7.*—Ascitic fluid incubated for 20 days. Feb. 1. Same procedure as in experiment 1. No effects produced.

*Experiment 8.*—Ascitic fluid incubated for 30 days. Feb. 9. Same procedure as in experiment 1. No effects produced.

## DISCUSSION.

The experiments described indicate that, with a highly active virus, 0.2 of a gram of the brain tissue taken from a recently paralyzed monkey will cause certain paralysis in a *Macacus rhesus* when injected by the intracerebral route. When, however, specimens are taken from the same portions of the brain from which the test fragment was removed and incubated in the ascitic fluid kidney medium, they still cause paralysis when similarly inoculated into a *Macacus rhesus* on the tenth or the twentieth day of incubation, but not on the thirtieth or the fortieth day. But the ascitic fluid adjacent to the brain fragments fails to set up paralysis in a *Macacus rhesus* when injected intracerebrally in quantities of 0.2 of a cubic centimeter after an incubation of ten, twenty, or thirty days.

The meaning of the experiments is clear. At the temperature of the thermostat, the ordinary virus of poliomyelitis survives in the autolyzing brain fragment containing it for a period of about twenty days, while it seems incapable of diffusing from the fragment into the surrounding medium in considerable quantity during the period of its active survival in the tissues.

The period of survival of the virus at 37° C. indicated by the experiments is almost exactly paralleled by the tests made by Levaditi<sup>4</sup> with fragments of infected intervertebral ganglia which he submitted to cultivation *in vitro*. Levaditi observed that the longest period at which the virus could still be detected in an effective state was twenty-one days, which period agrees exactly with our observations. Levaditi also noted that only the intervertebral ganglionic tissue, and not the surrounding plasma, was infectious. From this he concluded that the virus which he believed to have increased, multiplied only in association or symbiosis with nerve cells; but the observation is readily explained on the supposition that failure to detect the virus in the plasma was due to lack of its diffusion from the ganglionic tissue into the surrounding medium.

Hence the experiments performed for the express purpose of determining the limits of survival and degree of diffusion of the

<sup>4</sup> Levaditi, C., *Compt. rend. Soc. de biol.*, 1913, lxxiv, 1179; 1913, lxxv, 202.

virus at 37° C. have yielded results which coincide almost exactly with the results attributed by Levaditi to multiplication of the virus *in vitro* in association with proliferating tissue cells. It is highly probable that in Levaditi's experiments no multiplication of the virus whatever took place; and, moreover, that the discrepancy in respect to infectiousness observed between the ganglionic tissue and the plasmatic medium is explained, not by the proliferation of the virus within the former, but by the failure of diffusion into the latter.

Our experimental results are supported by observations recorded by Flexner and Noguchi,<sup>5</sup> which indicated that when no demonstrable growth of the microorganism has taken place in the ascitic fluid medium, the original tube, as well as the fluid in the early transfers, is wholly without power to incite poliomyelitis upon inoculation into monkeys.

The cultures, it will be recalled, are developed in the ascitic fluid kidney medium from fragments of infected brain tissue. From the original cultures obtained, subcultures are made by transferring after an inoculation of one or two weeks quantities of the ascitic fluid varying in amount from 0.2 to 0.5 of a cubic centimeter. The possibility of transferring in this way from one tube to another a certain quantity of the original virus of poliomyelitis contained within the brain fragment has always been considered. On the other hand, it has been pointed out that with each successive transfer, so rapid a dilution of any original virus present would doubtless take place that the fluid would soon lose infectious power from this source.<sup>6</sup> It now, however, appears that the quantity of virus which diffuses from the brain fragment into the ascitic fluid is so small that it may be disregarded from the outset.

Similar considerations relate to the survival of the virus in the brain tissue itself employed for the culture medium. Since the period of this survival is only about twenty days, cultures which have been incubated for weeks or months, and which are still active, cannot owe their infective power to the original virus, but must owe it to the multiplied microorganism. Moreover, when the

<sup>5</sup> Flexner and Noguchi, *loc. cit.*

<sup>6</sup> Flexner, Noguchi, and Amoss, *loc. cit.*

additional fact is taken into account that the successfully inoculated cultures were often many generations removed from the brain fragment from which they were derived, and in one instance had remained in the thermostat for a period exceeding one year, further consideration need hardly be devoted to the possibility that the experimental poliomyelitic infection is attributable in part to the coöperation of unchanged virus and culture of the minute micro-organism.

#### CONCLUSIONS.

The ordinary virus of poliomyelitis present in aseptically removed brain tissue of paralyzed monkeys survives in an ascitic fluid kidney medium at the temperature of 37° C. for a period of at least twenty, but not of thirty days.

Under the conditions of moderate anaerobiosis, the minute micro-organism cultivated from poliomyelitic tissues tends not to develop in cultures from the brain tissue; hence its presence does not complicate the survival test.

The diffusion of the ordinary poliomyelitic virus from a non-comminuted fragment of brain tissue into a surrounding medium of ascitic fluid is so slight as not to be detectable by inoculation experiments conducted with usual quantities of the fluid.

The specific effects of the microörganism cultivated from poliomyelitic tissues are not caused by an admixture in the cultures of the ordinary virus of poliomyelitis; hence they must be caused by the pathogenic action of the microörganism itself.

The minute microörganism is therefore to be regarded as the specific microbic cause of epidemic poliomyelitis.



## THE RAPID PRODUCTION OF ANTIDYSENTERIC SERUM.\*

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*(From the Laboratories of The Rockefeller Institute for Medical Research.)*

A side issue of the European war has been the unexpected and great demand for certain therapeutic sera. Among those for which a sudden demand has arisen is the antidyenteric serum, of which in the recent past large quantities have not been needed. It is doubtful whether the serum was being produced in notable quantities in any of the countries involved in the war, so that when dysentery appeared among the troops engaged in the western campaign, supplies of the serum were sought outside.

Among the early activities of The Rockefeller Institute was included a systematic investigation,<sup>1</sup> in the eastern part of the United States, of the bacteriology of acute dysentery and related diseases of the intestine. An important incident of this investigation was the preparation and testing upon human subjects of the antidyenteric serum. Doubtless, it was the knowledge of these studies possessed abroad that led to the requests made upon The Rockefeller Institute for supplies of the serum. This demand, which was met inadequately by supplies then available, led us to undertake the improvement of the method of preparing the serum, with the particular object in view of reducing the period required for the immunization of horses.

The outlook for success in this endeavor was hopeful, since the experience of the past few years had shown that certain antisera could be prepared in small animals—the rabbit especially—in far shorter periods of time than had been supposed. We owe especially to the

\* Received for publication, March 1, 1915.

<sup>1</sup> Flexner, S., and Holt, L. E., Bacteriological and Clinical Studies of the Diarrhoeal Diseases of Infancy. Report of the Rockefeller Institute for Medical Research, New York, 1904.

studies of Fornet and Müller,<sup>2</sup> Bonhoff and Tsuzuki,<sup>3</sup> and Gay<sup>4</sup> and his pupils the discovery of the fact that the reinjection of antigenic substances at brief intervals leads to an intensification of the antibody formation. In this manner and through three successive daily injections of protein, bacterial or body cells, strong precipitating, agglutinating, and cytolytic substances have been evoked. We have applied the principles of this method to the immunization of a horse with several types of the dysentery bacilli, with the result to be described.

#### VARIETIES OF DYSENTERY BACILLI.

Two distinct groups of bacilli capable of causing dysentery are now distinguished. As far as is known, the members of the group bring about similar or indistinguishable intestinal lesions and clinical symptoms.<sup>5</sup> As regards ordinary morphological and cultural characters, the groups are also indistinguishable; while they are readily distinguished by their power to ferment certain carbohydrates and alcohols (especially mannite), and by the important fact that one group yields in the cultures a soluble toxin and the other does not.

The original of the dysentery bacilli is designated the Shiga<sup>6</sup> bacillus, from the name of the Japanese bacteriologist who first isolated and studied the dysenteric bacillus. It happens that the Shiga bacillus possesses limited fermentative power and produces a soluble toxin. Dysenteric bacilli identical with the Shiga bacillus have been obtained everywhere in the Eastern, Western, and Southern Hemispheres in which they have been sought by bacteriologists. Hence the Shiga bacilli constitute the original group of dysenteric

<sup>2</sup> Fornet, W., and Müller, M., *Ztschr. f. biol. Techn. u. Method.*, 1908, i, 201.

<sup>3</sup> Bonhoff, H., and Tsuzuki, M., *Ztschr. f. Immunitätsforsch., Orig.*, 1909-10, iv, 180. Tsuzuki, M., *ibid.*, p. 194.

<sup>4</sup> Gay, F. P., and Fitzgerald, J. G., *University of California Publications in Pathology*, 1911-14, ii, 77. Gay, F. P., *Ergebn. d. Immunitätsforsch., exper. Therap., Bakteriolog. u. Hyg.*, 1914, i, 231. Locke, E., *University of California Publications in Pathology*, 1911-14, ii, 91.

<sup>5</sup> The question whether the Shiga bacillus is less often, and the Flexner group of bacilli more often present in sporadic cases of dysentery is still an open one; but members of both groups may cause epidemics.

<sup>6</sup> Shiga, K., *Centralbl. f. Bakteriolog., 1te Abt.*, 1898, xxiii, 599.

bacilli. They also compose an homogeneous group, since all the Shiga bacilli so far studied possess identical properties.

This is not true of the second group, which includes several quite well defined members. The first representative of this group was isolated by Flexner<sup>7</sup> from cases of acute dysentery occurring in the Philippine Islands. Hence in contradistinction to the Shiga group, it has become customary to speak of the other as the Flexner group. But, as already indicated, the latter is heterogeneous and contains two or more other varieties of closely related but yet distinguishable bacilli, sometimes designated as Y<sup>8</sup> and Strong.<sup>9</sup> None of the varieties in this group yields a soluble toxin; and while all show wider fermentation powers than the Shiga bacillus, yet the several members differ in that respect among themselves. Very recently Sonne<sup>10</sup> has described still another variant belonging in this group, which he cultivated from several cases of acute dysentery occurring in Copenhagen.

But the most important distinguishing feature exhibited by the dysentery bacilli relates to their antigenic or immunity properties. By employing especially the agglutination test, and to a less extent the test of protection, the Shiga and Flexner groups are more sharply, and the several members of the latter group less sharply separated from one another.

As regards the practical question of the specific therapy of dysentery, the most important consideration is that of protection. It is manifestly impossible to undertake to discriminate in many instances among cases of dysentery occurring sporadically in endemics or epidemics, the particular dysenteric bacillus causing the infection before applying the serum treatment. Two ways of proceeding are, therefore, indicated: first, to determine whether an antidysenteric serum prepared with the bacilli of one group protects against the bacilli of the other group; and second, to ascertain whether an effective polyvalent antidysenteric serum suitable for use irrespective of the type of infecting bacillus can be prepared.

<sup>7</sup> Flexner, S., *Philadelphia Med. Jour.*, 1900, vi, 414.

<sup>8</sup> Hiss, P. H., Jr., and Russell, F. F., *Med. News*, 1903, lxxxii, 289.

<sup>9</sup> Strong, R. P., and Musgrave, W. E., Report of the Etiology of the Dysenteries of Manila. Report of Surgeon General of the Army to the Secretary of War for 1900, Washington, 1900.

<sup>10</sup> Sonne, C., *Centralbl. f. Bakteriol., 1te Abt., Orig.*, 1915, lxxv, 408.

Indeed, it has long been known that the dysenteric bacilli possess both common and specific antigenic properties; a certain amount of cross-agglutination and protection exists which is more pronounced between the related members of the Flexner group than between the more widely separated Flexner and Shiga groups. Again, it has been found practicable to produce in a given horse by suitable methods of immunization antibodies for the two groups of bacilli, and in quantities which promise to suffice for practical therapeutic purposes. This fact is not only important as regards the treatment of cases of bacillary dysentery infected with a single strain,—the rule among cases ordinarily arising,—but is significant also in respect to the rarer instances of mixed strain infection, in which more than one member of the Flexner group, or in which the Shiga bacillus and a member of the Flexner group coöperate in causing the lesions and other effects of the disease.

#### RAPID PREPARATION OF POLYVALENT ANTIDYSENTERIC SERUM IN HORSES.

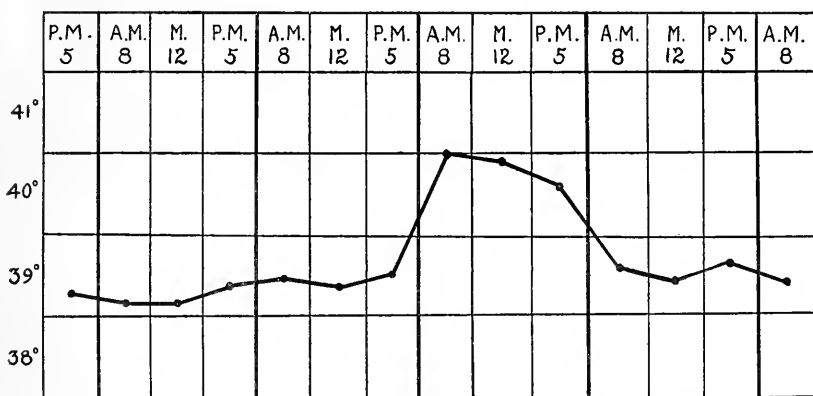
Hitherto horses have been injected with cultures or soluble products of the dysenteric bacilli over long periods of time. The injections have been made chiefly into the subcutaneous tissues, rarely into the veins. The severe reactions which intravenous injections often cause limited that method of immunization. The subcutaneous injections, on the other hand, are more readily carried out, but at the stage of the immunization process when large quantities of culture—or, in the case of the Shiga bacillus, soluble toxin—must be injected, large infiltrations may arise which sometimes ulcerate and depress the physical condition of the animals. The process of immunization is then halted until the ulcer heals and the general condition improves, entailing a loss of time; but in some instances a state of cachexia arises, leading to loss by death. By employing the subcutaneous method of immunization, an active antiserum can be prepared in from about nine to twelve months.

The process can be abbreviated by employing the method of successive intravenous inoculations over brief periods of time, with intervening periods of rest. The principles of the method are those developed by Fernet and Müller, Bonhoff and Tsuzuki, and Gay and

his pupils. By injecting successively the several varieties of dysenteric bacilli, a serum possessing high agglutinative and protective value has been secured in a period measured by weeks rather than by months.

*Cultures.*—The cultures employed were made upon agar-agar slant surfaces in tubes 15 by 160 mm. in size. The cultures were employed after twenty-four hours of incubation at 37° C., and the growth in each tube was suspended in 2 c.c. of 0.8 per cent. salt solution, from which measured quantities were taken for intravenous injection. The several members of the Flexner group and several strains of the Shiga bacillus were employed for injection.

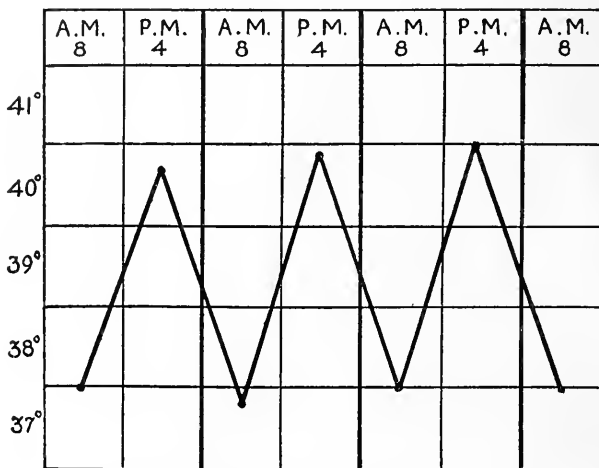
*Injection.*—Blood having been drawn in order to secure a sample for the control tests of agglutination and protection, the preliminary injection of cultures was made with 1 c.c. of the suspension of Flexner bacilli which had been heated to 60° C. for thirty minutes. The febrile reaction was slight. Twenty-four hours later 5 c.c. of the suspension were injected, which was followed by a sharp reaction (text-figure 1). The third injection was deferred forty-eight



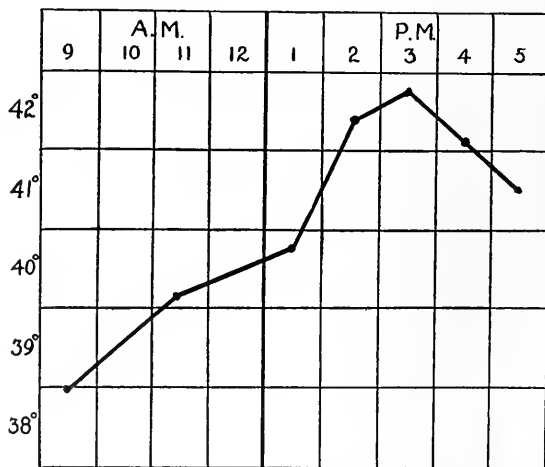
TEXT-FIG. 1. Febrile reaction to Flexner bacilli killed by heat.

hours, until the temperature again reached normal, when 5 c.c. of the suspension were again injected. After an interval of seven days, living cultures were substituted for the heated ones. The febrile reactions following the injection of four loops of the suspension on the first, ten loops on the second, and twenty loops on the third day were negligible. At the third inoculation suspensions of living Shiga bacilli were injected. The dose was four loops on the first, ten on the second, and thirty on the third day. From this time on, Flexner and Shiga bacilli were inoculated alternately on three successive days with intervening rest intervals of seven days, the doses being chosen so as to produce a sharp febrile reaction which subsided in twenty-four hours. By adjusting the

doses with greater care, the febrile curve was rendered typical (text-figure 2). As a rule, the dose of a culture which was injected on the third day of the last period was employed for injection on the first day of the next period. The



TEXT-FIG. 2. Typical febrile reaction from three injections on successive days.



TEXT-FIG. 3. Typical febrile rise and fall following each single injection.

increase in dosage was made by quite regular increments; namely, by fractions of  $+\frac{1}{8}$ ,  $+\frac{1}{5}$ , etc., of the quantity injected on the preceding day. It was ascertained that twenty loops of the culture weighed about 0.15 gm. When

the quantity of culture injected once exceeded twenty loops, the measurement of the suspension was made in graduated pipettes. From time to time blood was drawn for titration; it was taken just preceding a new series of inoculations, or, in other words, seven days after the last inoculation of the preceding series.

*Temperature.*—The temperatures were taken about one hour before each injection. It was noted that the maximal febrile point was reached about six hours after inoculation, after which the descent began, the normal being reached usually at about the end of the twenty-four hour period, which was the result strived for. The inoculations were made at about 9 A.M., and the temperatures were taken the fifth, sixth, and seventh hours afterwards, or until the figure indicated that the maximum had been reached and decline had begun (text-figure 3).

#### IMMUNITY VALUE OF THE ANTISERUM.

The degree of immunity produced by the several successive inoculations was ascertained by titrating the antiserum at intervals against the Shiga and Flexner groups of bacilli in respect to (a) the agglutination, and (b) the protective value.

*Agglutination.*—The titer of the horse serum before inoculation was begun for the several varieties of bacilli employed for injection is shown in table I. The Flexner group, as was previously known, is more readily agglutinated than the four strains of Shiga bacilli employed, although the Flexner D proved inagglutinable in the unheated serum.

TABLE I.  
*Agglutination Titer of Normal Horse Serum.*

Variety of dysentery bacilli.	Limits of agglutination.	
	Unheated.	Heated to 56° C.
Flexner group {	Flexner .....	1:120
	Strong .....	1:120
	Y .....	1:120
	Flexner D. ....	0
	Shiga 293 .....	1: 20 (partial).
	Shiga G. ....	1: 20 (partial).
	Shiga-Kruse .....	1: 20
	Shiga K. ....	1: 20 (partial)

The rapidity in the rise of the agglutination titer after each successive series of three injections of the bacilli is shown in table II. The values given are for complete agglutination in serum not inactivated by heat. Heating the serum to 56° C. reduced the agglutina-

TABLE II.

*Periodic Test of Agglutination Value for Flexner and Shiga Groups of Bacilli.*

Variety of bacilli.	1st period. <sup>11</sup>	2d period.	3d period. <sup>13</sup>	4th period.	5th period.	6th period.	7th period.	8th period.	9th period.	10th period.
Flexner. . . . .	1:500	1:800	1:1400	1:1500	1:2000	1:2000	1:3000	1:4000	1:4000	1:4000
Strong. . . . .	1:400	1:800	1:800	1:1400	1:1800	1:1800	1:3000	1:4000	1:4000	1:4000
Y. . . . .	1:300	1:800	1:800	1:1500	1:1800	1:2200	1:2300	1:3000	1:5000	1:5000
Flexner D <sup>12</sup> . . .	0	0	0	0	0	0	0	0	0	0
Shiga 293. . . .	—	—	1:80	1:90	1:500	1:500	1:1000	1:1800	1:3000	1:4000
Shiga G. . . . .	—	—	1:70	1:100	1:150	1:200	1:600	1:700	1:1200	1:1500
Shiga-Kruse. . .	—	—	1:100	1:90	1:200	1:200	1:600	1:800	1:1000	1:1200
Shiga K. . . . .	—	—	1:80	1:150	1:150	1:200	1:600	1:700	1:1000	1:1200

tion value by half or more. The values, moreover, are given for each bleeding period, which correspond with each successive set of inoculations of the bacilli. Hence the effect of each series of three injections can be followed. In the first period, killed bacilli of the Flexner group were injected; subsequently only living bacilli were employed. The effect of the first set of injections of the Shiga bacilli is seen in the third period. The rise in agglutinins for the Flexner group is more pronounced than for the Shiga group of bacilli. The inequalities as measured by the several varieties in each group are small, except in the culture denominated Flexner D, which appears to be inagglutinable.

*Protection.*—The therapeutic value of the serum was tested experimentally in guinea pigs and rabbits. The anti-infectious power was determined in the former and the antitoxic power for Shiga toxin in the latter animals.

The method employed was to mix suspensions of the living cultures with dilution of the serum or of Shiga toxin with serum, and to incubate the mixtures for one hour at 37° C., after which they were injected. The injections were made into the peritoneal cavity of guinea pigs weighing about 225 gm., and into the ear vein in rabbits weighing 1,250 to 1,500 gm.

The bacilli were cultivated on slant agar surfaces and the growths were of twenty-four hours' duration. The suspensions were made in sterile normal salt solution. The Shiga toxin consisted of a growth three days old of the Shiga bacillus in sugar-free bouillon containing calcium carbonate. The bacilli were killed with ether. The ether having been removed, the bouillon culture was passed through hardened filter paper and preserved in the refrigerator. Ether was found superior to phenol for killing the bacilli. It does not injure the toxin.

<sup>11</sup> After inoculation of cultures killed by heat.

<sup>12</sup> Probably inagglutinable.

<sup>13</sup> The first inoculation of Shiga bacilli given in previous period.



The protective value of the antiserum was compared with that of normal serum withdrawn from the horse before inoculation was begun. The effects of the normal serum are shown in table III.

TABLE III.  
*Anti-Infectious and Antitoxic Value of Normal Horse Serum.*

Flexner bacillus.			Shiga bacillus.			Shiga toxin.		
M.l.d.	Serum.	Result.	M.l.d.	Serum.	Result.	M.l.d.	Serum.	Result.
2	0.5 c.c.	Recovered	2	0.5 c.c.	Recovered	4	0.5 c.c.	Died. <sup>14</sup>
2	0.5 c.c.	Recovered	2	0.4 c.c.	Died	4	0.3 c.c.	Died.
2	0.2 c.c.	Died	2	0.3 c.c.	Died	4	0.2 c.c.	Died.
2	0.2 c.c.	Died	2	0.2 c.c.	Died	4	0.1 c.c.	Died.
2	0.1 c.c.	Died	2	0.1 c.c.	Died			
2	0.1 c.c.	Died						
2	0.05 c.c.	Died						
2	0.05 c.c.	Died						
2	0.01 c.c.	Died						
2	0.01 c.c.	Died						
2	0.008 c.c.	Died						
2	0.008 c.c.	Died						

The anti-infectious power of the normal serum was determined with two minimal lethal doses of the cultures, and the antitoxic power with four minimal lethal doses of the toxin. In the former instance, the total volume of each of the mixtures was two, in the latter one cubic centimeter.

Normal horse serum exerts slight protection only against the infectious or toxic effects of the dysentery bacilli. The limit of activity for two minimal lethal doses of the living cultures and for four minimal lethal doses of Shiga toxin is about 0.5 of a cubic centimeter of the normal serum.

With this value are to be compared the values of the serum after ten weeks' immunization which are shown in table IV. For the corresponding Shiga and Flexner bacilli it is 0.008 and for the Shiga toxin 0.003 of a cubic centimeter.

The protective and agglutination values resemble each other in appearing early and rising rapidly in the course of immunization. In view of this rapid development the serum should be suitable for therapeutic employment in man at the end of eight or ten weeks' immunization. Doubtless the continuation of the inoculations

<sup>14</sup> Death delayed somewhat.

TABLE IV.  
*Anti-Infectious and Antitoxic Value of Immune Horse Serum.*

Flexner bacillus, <sup>15</sup>			Shiga bacillus.			Shiga toxin.		
M.l.d.	Serum.	Result.	M.l.d.	Serum.	Result.	M.l.d.	Serum.	Result.
2	0.1 c.c.	Recovered	2	0.1 c.c.	Recovered	4	0.5 c.c.	Recovered.
2	0.05 c.c.	Recovered	2	0.05 c.c.	Recovered	4	0.4 c.c.	Recovered.
2	0.02 c.c.	Recovered	2	0.01 c.c.	Recovered	4	0.2 c.c.	Recovered.
2	0.01 c.c.	Recovered	2	0.008 c.c.	Recovered	4	0.1 c.c.	Recovered.
2	0.008 c.c.	Recovered	2	0.003 c.c.	Died	4	0.008 c.c.	Recovered.
2	0.008 c.c.	Died	2	0.001 c.c.	Died	4	0.005 c.c.	Recovered.
2	0.005 c.c.	Died	2	0.0005 c.c.	Died	4	0.003 c.c.	Recovered.
2	0.003 c.c.	Died				4	0.002 c.c.	Died.
2	0.002 c.c.	Died				4	0.001 c.c.	Died.

would result in still further accumulation of specific antibodies. But it seems safe to begin regular bleeding of the horses treated in the manner described at the tenth week of the immunization process and to carry out regular bleeding with intervening inoculation from that period on.

#### SUMMARY.

Antidysenteric serum can be safely prepared in the horse by the method of three successive intravenous injections of living cultures or toxin with intervening rest periods of seven days.

When this method of immunization is employed, the specific antibodies responsible for agglutination and protection appear early and rise rapidly.

By inoculating alternately living dysentery bacilli belonging to the Shiga and Flexner groups a polyvalent serum of high titer may be prepared.

A polyvalent serum so produced should be suitable for the therapeutic treatment of acute bacillary dysentery, irrespective of the particular strain or strains of the dysentery bacillus causing the infection.

An effective antidysentery serum suitable for therapeutic employment in man can be prepared in the horse in about ten weeks.

<sup>15</sup> The experiment was done in duplicate.

A STUDY OF BARLOW'S DISEASE EXPERIMENTALLY  
PRODUCED IN FETAL AND NEW-BORN  
GUINEA PIGS.\*

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PLATE 38.

The relation between the organisms of the mother and offspring during the period of pregnancy has been the subject of thorough investigation. In both clinical and experimental pathology a number of studies, through a varied series of influences on the maternal organism, throws light upon the secondary influences on the fetal body. The agents employed to produce disease have usually been chemical, physical, or parasitic in character, but investigations as to the influence of dietetic changes upon pregnancy are limited in number. I may mention here particularly the dietetic experiments with strontium made by Lehnerdt<sup>1</sup> on pregnant rabbits. He succeeded in producing in young fetuses a disease of the osseous system, which exhibited macroscopically numerous fractures and infractions of the long tubular bones, and microscopically an increase in the osseous tissue, with extensive new formation of osteoid tissue and greatly impeded reabsorption.

In the present paper I wish to describe certain experiments made upon guinea pigs during pregnancy. The constant character of the frequently fatal disease that was produced in guinea pigs within a period varying from ten to twenty-eight days by a diet consisting exclusively of oats and water in Holst and Frölich's experiments<sup>2</sup> coincided closely both macroscopically and microscopically with Barlow's disease in man.<sup>3</sup> This, therefore, seemed to give an

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<sup>1</sup> Lehnerdt, F., *Beitr. z. path. Anat. u. z. allg. Path.*, 1909, xlv, 468; 1910, xlvii, 215.

<sup>2</sup> Holst, A., and Frölich, F., *Ztschr. f. Hyg. u. Infektionskrankh.*, 1912, lxxii, 1.

<sup>3</sup> Ingier, A., *Frankfurter Ztschr. f. Path.*, 1913, xiv, 1.

opportunity of studying the possible changes in the osseous system of the fetus produced by a scorbutic diet, and also of obtaining some insight into the biological relation existing between mother and offspring. These studies might lead to conclusions with regard to the relation of the various constitutional osseous diseases of unknown etiology that occur spontaneously in man, the principal symptoms of which are dystrophy of the bone-forming elements.

The following diseases will be considered: osteogenesis imperfecta (congenital osteopsathyrosis), rachitis, and osteomalacia.

Before describing my experiments I wish particularly to emphasize the fact that the diet was tried on mature animals and on quite young ones, and also at various stages of pregnancy. The investigations showed that the stage of embryonic development as well as the resisting power of the pregnant animals is of importance, since in the experiments mainly older animals and those during the latter half of the period of pregnancy gave birth to living and apparently mature offspring, whereas experiments performed on young animals and those in the earlier stages of pregnancy invariably resulted in premature birth or in the birth of a dead fetus.

Thirty-three of my experiments were successful. They may be divided into three groups. Group I comprises the fetuses that were born early, prematurely or by miscarriage, within ten days after the beginning of the new diet. The second group comprises fetuses that were born prematurely or fetuses in an advanced stage of development between the tenth and twenty-fifth days. Group III comprises the young ones that were apparently fully developed and living, born within twenty-three days after the beginning of the experiment, which survived and were given a scorbutic diet; *i. e.*, milk from scorbutic mothers and later on oats and water.

The first group contains the litters of twelve animals. The fetuses had been alive for five to ten days after the experiment began. The litters consisted mainly of two, in some few cases of three or four animals. The young were either still-born or were found as dead fetuses in the uterus of the dead mother.

It appears that animals are more sensitive to changes in diet during pregnancy than at other times, since a comparatively large percentage of the mothers died in the early stages of the experiment.

All the fetuses showed a low stage of development. The weight varied from seventeen to fifty-five grams (the latter occurring once only), and the average was between twenty and thirty grams.

Macroscopically the bones presented nothing remarkable. Microscopic examination revealed no changes in the skeletons even of the longest living fetuses, with the exception of hemorrhage and thrombosis in the marrow.<sup>4</sup>

The second group comprises the litters of seventeen animals that had been fed during pregnancy on a scorbutic diet for twelve to twenty-five days. These cases present great variety. The degree of scorbutic change in the fetus was not proportional to the length of the period of intra-uterine dieting with scorbutic food. These changes, too, as regards intensity were considerably smaller than those in the skeleton of the mother. Thus, for instance, in one case after a diet of oats and water for twenty-two days, the offspring being alive and apparently fully developed (weight seventy-one grams), only isolated hemorrhages in the marrow and scarcely any perceptible reduction of osseous substance could be discerned under the microscope; whereas in another case, also after twenty-two days of experimental diet, the two young ones were born with pronounced Barlow's disease. They were still-born, undersized, apparently belonging to the first half of the period of pregnancy, although at the commencement of the experiment the stage of pregnancy, by palpation of the abdomen, seemed to be fairly advanced. It is, however, an extremely difficult matter to determine the exact stage of a litter.

Figures 1 and 2 show the microscopic structure of this case. They are microphotographs of one of the upper extremities. As in the other instances, there is no change in the cartilage. In the proliferative zone, the columnar zone, and the calcified layers of the cartilage, we find conditions that come within the scope of physiological variation. Destruction of cartilage has taken place almost symmetrically, by the formation of long columns of calcified ground substance that are partially connected into a network. These pillars sometimes continue subchondrally to the center of the bone. No

<sup>4</sup> As regards the purely technical aspect of the experiments, it may be stated that the bones were hardened in formol immediately after death, and subsequently partially decalcified in Müller's fluid. The method of staining employed was principally hematoxylin and eosin.

traces of osseous tissue can be found in the central or subchondral parts or in the zone of primary marrow spaces. Osteoblasts are also absent. The wide medullary spaces and the central medullary cavity are filled with structural marrow ("*Gerüstmark*"), consisting of fine fibers, spindle and stellate cells (medullary substance), in which only scattered specific osteomyelon cells are to be found.

The corticalis consists of a thin, osseous strip scarcely wider than a single row of osseous units where no apposition of osteoid tissue is discernible. In the center of the humerus a complete oblique fracture has taken place. The ends of the fracture have been displaced. The distal end has slipped along the outer surface of the upper one, and lies close to it, while the proximal end is wedged into the loose tissue of the cubital angle. At this spot copious and recent hemorrhage had taken place. We find here, moreover, numerous pigment cells. The periosteum, although torn, was not detached from the osseous tissue by the hemorrhage. At the seat of fracture there was hyaline degeneration of the periosteum and reaction was not discernible. At this point in the bone marrow connective tissue was beginning to be loosened.

In all the bones examined in this case abnormal length and persistency of the calcified columns of cartilage, deficiency of bone formation and medullary substance were found, and also fresh fractures and infractions in nearly all the long cylindrical bones. Even the scapula was fractured and only the ribs were whole. In all cases the hemorrhages were of small extent and few in number. Osteoclasts were very rare.

In reviewing these facts we must note that we are dealing with a case where the fetus corresponded to an earlier stage of development than could be expected from the time during which the pregnancy had been observed and the experimental dieting had lasted. The fetus, both macroscopically and microscopically, was backward in development.

On microscopical examination a perfectly normal appearance of the cartilage was revealed. The region of the endochondral ossification showed a deficiency of bone formation, since neither old nor recent osteoid tissue was discernible. The marrow was a typical medullary substance with few osteomyelon cells. As a rule, hemor-

rhages were rare. The periosteal formation was also diminished. Furthermore, if we take into consideration the fact that in numerous bones fractures and infractions of an undoubted intra-uterine origin were found, with merely a slight reaction from the periosteum and medulla, it is evident that we have all the symptoms of a fully developed case of Barlow's disease.

The second fetus of the case in question showed the same signs of the disease in an advanced stage. It is unnecessary to discuss the remaining cases of this group, as nearly all exhibited the disease to a lesser extent.

The litters comprised in group II were also premature. The weight was somewhat more than that given above; as a rule, between twenty-five and thirty-five grams. These figures were rarely exceeded. Thus in two cases in which birth occurred after twenty-one and twenty-two days respectively, the weights of the fetuses in one were sixty-four and sixty-eight grams, and in the other fifty-five and thirty-three grams. Of these litters only the latter exhibited marked scorbutic changes in the bones, in the form of medullary substance, sparse hemorrhage in the marrow, and markedly diminished osseous formation; whereas in the first litter, with the exception of loose teeth no changes were discernible in the skeleton, even with the aid of a microscope.

In the second group, too, the death of the mother took place comparatively often, five times out of seventeen, on the twelfth to fifteenth day of the experimental diet, and long before the expected time of delivery. The fetuses which were found in the uterus of the dead mothers all showed a stage of development approximately corresponding to the end of the first half of the period of pregnancy. Even after a lapse of twelve days these fetuses exhibited traces of a slight diminution of osseous formation. It is noteworthy that in one case a fetus showed advanced symptoms of Barlow's disease, exactly as described above, as early as the fifteenth day of experimental dieting; yet in this instance I observed the infraction of the corticalis in the subchondral zone, as is typical of Barlow's disease. The seat of infraction was the inner and upper end of the ulna, where it touches the head of the radius, and this fact may be of

importance as regards the origin of the fracture through intra-uterine movement.

Regarding the atypical seat of the fractures and infractions in these intra-uterine cases, they may perhaps be explained by assuming that the seat of fracture has moved towards the center by reason of the later growth of the bones. The pigment cells at the seat of fracture show that mainly older fractures are under consideration. The fact that in the case described an oblique fracture has taken place, agreeing entirely with one occasioned by indirect violence—by forcible bending—makes it probable, however, that the seat of the fracture is not dislocated by growth of the bone, but has taken place in the spot where it was found. The abundant fresh hemorrhage in this case also strengthens our argument. This difference in the seat of the fractures may be explained as follows: The predisposing factor of typical subchondral fractures in Barlow's disease may be sought in the circumstance that in this newly formed osseous zone a locus minoris resistentiæ is formed by the disease, which is, however, not the case with intra-uterine fractures, the corticalis being equally thin everywhere. It is noteworthy that there are no fractures of the ribs.

The origin of an intra-uterine fracture may be explained in this disease as well as in osteogenesis imperfecta as a mechanical action caused by contraction of the muscles (intra-uterine movements).

The results of my experiments prove beyond doubt that we can produce experimentally in the guinea pig embryo during its fetal existence a disease characterized by all the main features of true Barlow's disease.

In the third group of my experiments, comprising four cases from a later stage of pregnancy, the fetal skeleton, having reached a further development, displays a more marked picture of the disease, which resembles the scurvy of the adult animal still more. The secondary changes, however, are not so pronounced as those found in adult guinea pigs that were fed on oats and water until death.

The cases comprised in group III are all remarkable for the high degree of development of the fetus. They all appear to be fully developed and alive at birth. The weight was fifty-five to sixty-five grams.



The duration of experimental dieting, intra-uterine and extra-uterine, was as follows:

- Case 1, 23 dys. intra-uterine, 5 dys. extra-uterine (mother's milk, oats, and water).  
Case 2, 13 dys. intra-uterine, 16 dys. extra-uterine (mother's milk, oats, and water).  
Case 3, 8 dys. intra-uterine, 7 dys. extra-uterine (mother's milk, oats, and water).  
Case 4, 14 dys. intra-uterine, 5 dys. extra-uterine (mother's milk, oats, and water).

The most remarkable feature of these cases was the slight development of the secondary changes in the zone of ossification, which is so marked in human cases of Barlow's disease. This results from the slighter hemorrhage in the bone marrow, and in consequence the lesser disturbances of the circulation in the zones of subchondral and primary marrow spaces. In these cases, too, even after sixteen days of extra-uterine life, there results a symmetrical destruction of cartilage, with no changes in the cartilage, even in the bones where usually we find the highest degree of change; *viz.*, the ribs and extremities of the knee joints. The newly formed columns of calcified primary cartilagenous substance are more scarce than in normal animals; they are usually short and thick; only a few continue subchondrally as more substantial osseous columns. The subchondral spongiosa consists of scattered, isolated, somewhat small calcareous columns, on which only small quantities of osseous substance are deposited. Neither here nor in the zone of primary marrow spaces are there any traces of osteoid tissue. Osteoblasts are also rare, and exclusively spindle-like in shape. The corticalis is very thin, especially in the subchondral zone.

In nearly all cases we can trace subchondral infractions of the corticalis, and also isolated broken cartilage columns. These fractures are most pronounced in the offspring of the second case, which were influenced by the injurious diet for thirteen days *intra uterum*, and sixteen days *extra uterum*, and lived longest of all the animals experimented upon.

In the ribs, and particularly in the tibiæ, there were subchondral fractures with extensive, fresh hemorrhages in the periosteum and bone marrow. The circumstance that the epiphysial centers of ossification of the bones contained extensive fresh hemorrhages in the marrow proves that the latter were not only of traumatic nature, but

also were due to a hemorrhagic diathesis. Also the characteristic formation of medullary substance in the subchondral zone in all cases leads us to the conclusion that in these cases the changes in the skeleton increase not only with the duration of experimental feeding, but first and foremost with the mechanical strain on the skeleton of the extra-uterine animal. This seems to be directly confirmed by the circumstance that infractions and extensive hemorrhages were lacking in one of the offspring of case I of this group, which was killed immediately after birth, after twenty-three days of intra-uterine life, while diminished formation of bone and formation of medullary substance were discernible.

#### DISCUSSION.

From the experiments here described it is evident that a disease of the skeleton may be produced not only in the pregnant guinea pig, but also in the fetus, by feeding the mother exclusively on oats and water. The condition thus produced is characterized as Barlow's disease by the diminished or even non-existing formation of bones, by the existence of hemorrhages in the bone marrow, by the formation of medullary substance, and by the occurrence of fractures.

During the first half of the period of pregnancy the lack of the conditions necessary to sustain life, occasioned by the special diet, is so marked that the animals die almost at the beginning of the period of experiment, and in these cases microscopic examination of the skeleton shows only hemorrhages and thrombosis in the bone marrow.

In group II of my experiments I succeeded in keeping the animals alive for a somewhat longer period. In these cases, too, the fetuses were undeveloped, apparently belonging to the first half of the period of pregnancy, and were frequently still-born. In these cases I found, besides hemorrhages in the marrow, a more or less marked reduction of bone formation, in some instances complete cessation, a development of typical medullary substance and even undoubted intra-uterine fractures.

More marked secondary changes of the bones occurred after birth only by the consequent mechanical strain on the skeleton, the animals

being deprived continuously of the necessary materials for normal development of the bones, by dieting them on milk from a scorbutic mother, and on oats and water only. These secondary changes consisted in larger fractures in the epiphysial zone, indications of a more or less pronounced zone of decay, more extensive hemorrhages in the periosteum and bone marrow, and a reaction of connective tissue by the osteomyelon, corresponding exactly with a fully developed case of Barlow's disease as seen in adult animals after twenty-eight days of an oat diet. No case, however, showed a more marked disturbance of the endochondral reduction, or change in the cartilage proper.

On comparing the cases of Barlow's disease in the mother animal and in the offspring, it is seen that the life of the fetus is not of longer duration than the life of the mother, and this is true in cases fed with scorbutic food during the intra-uterine life, as well as in cases in which this period is succeeded by a period of postnatal scorbutic feeding. None of the fetuses were alive for more than twenty-eight days; that is, they have the same duration of life as the adult animals under the same conditions. On this point, mothers and young animals give the same results.

On the other hand, both macroscopic and microscopic changes in the skeletons of the fetus, although pronounced, were evidently less than those in the adult animal. Thus the zone of decay was always lacking and, accordingly, the secondary changes in the cartilage. Therefore the offspring that were born alive were lively and active. Several points must be considered in order to explain this circumstance. I am inclined to believe that the first cause is the vital independence of the fetus in the uterus. In the case of diseases occurring spontaneously, as well as in injury occasioned by experiment to pregnant individuals, the damage to the organism of the fetus is always less than to that of the mother. We know that the state of nourishment of the mother does not in the least correspond in normal pregnancy with that of the new-born child. On the other hand, Goldmann's<sup>5</sup> experiment with vital staining proves directly that during the development of the fetus there is established a "kind of

<sup>5</sup> Cited by Wolff, B., in von Meyer and Schwalbe, *Studien zur Pathologie der Entwicklung*, Jena, 1913, i, 50.

center," to which "certain nutritious substances, *e. g.*, glycogen and fat, are directly attracted from the maternal organism."

Moreover, remembering that pregnant animals show a more advanced state of the disease in their bones at an earlier period of defective diet than is the case with non-pregnant animals, it appears that by a reduction of the amount of the essential components of diet, the fetus absorbs a comparatively high percentage of those substances, by reason of its superior intensity of assimilation.

Another important cause of the lesser development of osseous changes in the fetus is no doubt to be found in the circumstance that its protected position in the uterus wards off all but a few minor mechanical influences. This is shown again by the circumstance that very few days of extra-uterine existence are sufficient to produce marked symptoms of Barlow's disease even in cases where at birth the disease occurs only in a latent state. I was able to trace only slight disturbances of the skeleton in the case of young animals of the third group that were killed immediately after birth.

In these experiments we notice the high percentage of premature births and of still-born litters. I do not know whether any corresponding phenomenon has been observed in human beings during scorbutic epidemics, nor are there any accounts of cases of congenital Barlow's disease in the medical literature; and considering the long period of development of this disease in human beings and the rapid recovery caused by suitable diet, this circumstance is not remarkable. Judging from the complete correspondence of the disease in human beings and in guinea pigs, we must assume that such cases may exist. On the other hand, Cheadle and Poynton<sup>6</sup> mention scorbutic offspring whose mother suffered from the same disease.

With reference to the etiology of Barlow's disease when produced *intra uterum*, there is no doubt that unsuitable diet occasions a species of pathological assimilation that equally influences the organism of both mother and offspring. We may presume that a certain substance is lacking in the food, which substance is equally essential to both mother and infant. Yet even these experiments disclose

<sup>6</sup> Cheadle, W. B., and Poynton, F. P., in Allbut, C., and Rolleston, H. D., *A System of Medicine*, 1909, v, 898.

nothing as to the real nature of this action, whether directly on the bone-forming elements of the osteomyelon or by way of some inner gland secretion, or perhaps by a species of intoxication. It has occasionally been shown that by an insufficiency in the mother of a gland with inner secretion, a vicarious hypertrophy of the same gland occurs in the fetus. Thus the thyroid gland in the fetus is hypertrophied when that gland is removed from the mother (Halsted and others).

We may imagine something of the same kind in the case before us, but I have found nothing macroscopically that would lead to such an interpretation. No microscopic examination, however, has been undertaken with this in view. Undoubtedly the fetus cannot remain alive for a longer period than the adult animal, nor can it defend itself against the development of the disease.

At the same time, experiments made for the purpose of determining a possible failure of some secretive gland in adult animals gave no positive results. By the addition of various preparations of glands influencing the growth of bones (thyroid, hypophysis, thymus, parathyroid) to the oats and water diet, no positive results were obtained in a single case, either *per os* or parenterally. Nor did I succeed in influencing the disease by parenteral introduction of defibrinated blood from normally fed guinea pigs, in which presumably secreted substances from all the glands are present.

On the other hand, no experimenter has so far succeeded in a direct demonstration of the possible lacking substance in scorbutic diet. In this connection it is interesting to recall Schmorl's<sup>7</sup> investigations on Heubner's dogs, in which by a diet deficient in phosphates he found the symptoms of a disease that showed great likeness to Barlow's disease, but which could not be identified with it. Experiments which I have performed on adult guinea pigs point in the same direction, inasmuch as I did not succeed in checking the development of the scorbutic phenomena by adding phosphorated cod liver oil to the oats diet.

As regards the relation between the experimental results given here and the congenital disease osteogenesis imperfecta (congenital osteopsathyrosis) which occurs spontaneously in human beings, and

<sup>7</sup> Schmorl, G., *Arch. f. exper. Path. u. Pharmacol.*, 1913. lxxiii, 313.

which also is due to a diminution of the osseous development, it is clear even from the morphological and symptomatological discoveries in both instances that they differ essentially. The fact that I did not succeed in producing a disease resembling it, even by an alteration in the experiments whereby the injurious influences were less extensive, although allowed to continue for a longer period, is in accord with the above hypothesis. The circumstance is in full accord with the demands for a specific etiology of Barlow's disease, whereas the etiology of osteogenesis imperfecta is still unknown, whether occasioned by an injury to the ovum, or to the embryo at a very early stage.

By adding thirty grams of cabbage, boiled for one-half hour at a temperature of about  $110^{\circ}$  to  $120^{\circ}$  C., to the daily ration of oats, Holst and Frölich succeeded in keeping the animals alive for several months; when death occurred as the result of this diet, the animals showed scorbutic changes. As mentioned above, I failed to produce during this modification of the experiments an injury to the bone-forming elements coincident with their formation, which would assert itself in the later development of the skeleton. This diet also resulted in premature delivery; the fetus showed no particular changes in the skeleton; only lesions of scorbutic origin were observed.

These experiments, however, are of special interest on account of another disease of the bones which occurs in human beings during pregnancy; *viz.*, osteomalacia. Although exacerbation always occurs during pregnancy in this disease, I am not aware of any case in which a congenital osteomalacia or rachitic disease has been traceable in the fetus or in full term children of such mothers.

With respect to the total of the examples of congenital rachitis recorded in medical literature, even after eliminating osteogenesis imperfecta and chondrodystrophia fetalis, which were formerly regarded as fetal rachitis, the remainder consists essentially of cases based upon macroscopic observations. On the other hand, no authors, not even Schnorl in the course of his extensive examinations of the skeletons of human fetuses and the newly born, have so far succeeded in finding the pathognomonic aspect of the microscopic diagnosis of rachitis,—excessive periosteal superposition or thickening

and plane extension of the osteoid seams. Nor can the macroscopic appearance of intumescences at the limits of the cartilage be considered pathognomonic for rachitis, because the former exist as secondary symptoms in several osseous diseases; *e. g.*, rachitis, Barlow's disease, traumatic disturbances, and recently the "snuffles" in hogs.<sup>8</sup>

The circumstance that no child suffering from rachitis or osteomalacia has been known to be born of a mother suffering from osteomalacia during pregnancy, even when the disease has lasted throughout the full period of gestation, assuming that this factor will assert itself in future investigations, indicates that the two diseases are fundamentally different both as to their nature and their etiology. It is all the more noteworthy because Hart<sup>9</sup> claims to have discovered a case of rachitis among his cases of Barlow's disease during a series of experiments on monkeys, all of which had been put on the same scorbutic diet, and lived under the same conditions. He assumes a common etiology between Barlow's disease and rachitis.

We are thus concerned, on the one hand, as regards osteogenesis imperfecta with a disease in which an injury to the fetus may occur without any traceable illness in the mother, which seems to indicate a primary injury to the fetus. On the other hand, in osteomalacia we observe that a healthy fetus may be born of a constitutionally diseased mother. By the selective action of the placenta, that is, of the chorionepithelioma, the fetus is able, in the latter disease, to avoid the influence of the factors that are injurious to the mother.

While in the case of Barlow's disease the assumption, as an etiological factor, of a deficiency of some nutritive substance essential to the support of life as well as to the action of the bone-forming cells of the osteomyelon, would appear to be probable, we might perhaps in osteomalacia imagine a hormonal effect. The general relation between various secretive glands and the growth of the bones is well known. Thus, for instance, the disturbance of the function of the hypophysis is connected in some cases with acromegaly (Fischer and others), and in the same way the parathyroid glands are probably closely connected with the disturbances of osteomalacia (Erdheim, Todyo).

<sup>8</sup> Ingier, *Frankfurter Ztschr. f. Path.*, 1913, xii, 270.

<sup>9</sup> Hart, C., and Lessing, O., *Der Skorbut der kleinen Kinder*, Stuttgart, 1913.

## CONCLUSIONS.

1. Pronounced cases of Barlow's disease may be produced in the fetus as early as ten to fifteen days after the commencement of dieting pregnant guinea pigs with oats and water. There are wide individual variations. The scorbutic changes in the skeleton are greatest in the earlier embryonic stages. The fetuses of that period, with practically no exceptions, die and show marked traces of impeded growth.

2. Fetuses from the later period of pregnancy are born alive, and apparently fully developed, with comparatively slight changes in the osseous system.

3. Even a short extension of the period of extra-uterine dieting on milk from scorbutic mothers and later on oats and water is sufficient to change the latent scurvy into a highly pronounced case.

4. The fetus cannot be kept alive longer than the adult animal, about twenty-eight days, either by intra-uterine dieting alone or by combined intra- and extra-uterine dieting.

5. The mothers show signs of disease at an early period, and are more severely attacked than non-pregnant animals. Death also occurs comparatively often in the first period of gestation.

## EXPLANATION OF PLATE 38.

FIG. 1. General view of the humerus, ulna, and radius. At the limits of the ossifying cartilage in the humerus both metaphyses show a close network of calcified primary cartilagenous substance, with normal conditions in the cartilage; there is no endochondral formation of bone. The corticalis is thin throughout. In the middle of the bone there is a dislocated oblique fracture. The proximal piece is wedged into the loose tissues at the angle of the elbow and surrounded by hemorrhages. The periosteum shows hyaline degeneration at the seat of fracture, but no formation of callus. The marrow, which is typical structural medulla, contains a slight reaction of tissue at the fracture.

FIG. 2. The proximal extremities of the ulna and the radius, more highly magnified. Normal conditions in cartilage. (The black spot is due to a fault in preparation.) At the limits of ossifying cartilage, especially in the radius, is seen a close network of calcified primary cartilagenous matter, which continues in long calcified cartilagenous columns. In other sections the latter extend almost to the center of the bone. No endochondral formation of osseous matter is present. There is typical medullary substance throughout the bone. The corticalis is evenly thin, and the ulna shows in the middle of the bone an infraction of the corticalis with a slight thickening of the cambium layer at that point.





FIG.1.



FIG.2.

(Ingier: Barlow's Disease Experimentally Produced.)



# PURE CULTIVATION IN VIVO OF VACCINE VIRUS FREE FROM BACTERIA.\*

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PLATES 39 TO 50.

In spite of much effort no method has, up to the present time, been perfected by which vaccine virus can be propagated in a pure state, free from contaminating bacteria. The method of propagating vaccine virus universally practiced today consists in transmitting the virus from the skin of one calf to that of another. Although the inoculation of the virus is carried out under precautions as strictly aseptic as possible, the fresh product nevertheless yielded by the skin contains a not inconsiderable number of different bacteria derived from the skin surfaces, the air, etc.

The employment of glycerin as an elective germicide against the non-spore-bearing bacteria contained in fresh, or so called "green," vaccine pulp results in a great reduction both in number and variety of the contaminating microorganisms, without at the same time seriously impairing the activity of the vaccine virus (1). After contact with concentrated glycerin, in a refrigerator, ranging from one to three months, the virus usually is freed from most of the bacteria and becomes "ripe" for practical use in vaccination on human beings.

Different samples of the ripe vaccine preparations, as issued from various authorized sources, vary in their activities as well as in their germ contents. Among the organisms which may be encountered in such preparations are the following: streptococci, staphylococci, *Bacillus coli*, *Bacillus welchii*, *Bacillus xerosis*, *Bacillus subtilis*, and some other aerobic and anaerobic bacteria (2). The action of glycerin on bacterial spores, which may also be present in the pulp, is

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almost nil. Moreover, the prolonged action of glycerin tends gradually to reduce the activity of the vaccine virus itself, so that the fresh vaccine pulp forfeits some of its efficacy while undergoing the "ripening" process. At the end of the ripening process, which consumes from one to three months, the virus usually still contains a certain number of bacteria; bacteria-free virus is practically never produced by glycerination. But the residue of living bacteria, fortunately, is to all intents and purposes quite harmless and practically to be disregarded. And yet it is apparent that it is not only desirable to eliminate all bacteria from vaccine virus, but the saving of the time lost in "ripening" and the preservation unimpaired by glycerin of the full strength of the virus are also highly important considerations.

#### HISTORICAL.

The observations of Béraud (3), Weigert (4), Chiari (5, 6), Councilman, Magrath, and Brinkerhoff (7), and others (8) indicated that the variola virus localizes in different viscera and organs; the vaccine virus, on the other hand, according to Calmette and Guérin (9), and von Prowazek and Yamamoto (10), exhibits a high degree of affinity for pavement epithelium. When the vaccine virus is introduced directly into the blood or peritoneal cavity, it lodges in the epithelia of the skin and mucous surfaces, provided they are injured within a few hours of the inoculation, while in a few hours more it has completely disappeared from the circulation or the peritoneum. Should the epithelium not take it up, it is entirely lost.

In order to test the question whether still other epithelial cells take up the virus, Henseval and Convent (11) conducted in 1910 a series of experiments on the testes of rabbits. The virus that they employed contained staphylococci and *Bacillus subtilis*.<sup>1</sup> The effect was to produce some swelling, but no induration of the organ. Five to seven days after inoculation the testes were extirpated, emulsified in 66 per cent. glycerin, and tested on the shaved skin of rabbits. The activity was less than that of the original material. From the first testicular emulsion a second set of rabbits was injected; the swelling and induration produced was greater, but the activity of the emulsion was no stronger. No attempt was made either to free the vaccine of bacteria or to continue the testicular passages. Later, Paschen (12) injected vaccine (its bacterial content is not stated) into the testicle of a rabbit and noted the production of necrosis and cellular infiltration. As early as 1904 Tyzzer (13) attempted to obtain characteristic vaccinia lesions by inoculation of the testis of rabbits and a calf; the result was regarded as an acute inflammatory reaction. Von Prowazek and Miyaji (14) state that vaccine virus injected into the testicle of the rabbit can

<sup>1</sup> The number of contaminating bacteria in the three samples employed was 4, 10, and 25,000 per 0.01 c.c., respectively.

still be detected two days later, and that local necrosis of the tissue appears at the inoculation site. Nothing is said of the bacterial content.

That the testicle of the rabbit affords a favorable site for the multiplication of parasitic microorganisms can be inferred from the experiments of Parodi (15) and later of Uhlenhuth and Mulzer (16) with *Treponema pallidum*, of Nichols (17) with *Treponema pertenue*, and of a host of later workers with *Treponema pallidum* (18). I therefore turned my attention to the cultivation of pure strains of vaccine virus in the rabbit's testicle.

#### PURIFICATION OF VACCINE VIRUS PRIOR TO CULTIVATION.

At the outset and before describing the details of the experiments on the cultivation of vaccine virus it is necessary to point out a material difference between the employment of the rabbit's testicle for *Treponema pallidum* and for vaccine virus. In the case of the former the multiplication of the spirochætæ takes place slowly, so that the contaminating bacteria which are carried into the testicle with the inoculated fragments of syphilitic tissue may in time be suppressed by the bactericidal action of the vigorous tissues. On that account pure strains of the pallidum may be thus developed after a few passages. Vaccine virus, on the other hand, multiplies quickly, and the inflammatory reaction and necrosis of tissue which ensue quickly suppress the bactericidal process of the testicular tissues and place them in a favorable state for bacterial development. On that account the virus must first be freed from the bacterial contamination by other means.

*Preliminary Purification.*—Several antiseptic or disinfecting substances may be employed for reducing or removing contaminating bacteria. Glycerin is universally employed in practice to reduce the number, because of the small effect which it exerts on the virus itself. At low temperatures the antiseptic action is too weak to eliminate all bacteria, but at 37° C. in 60 per cent. strength all bacteria except spore-bearing species may be destroyed in a few days. Hence it is suitable for the purification when the virus is devoid of sporogenous bacteria. Addition of 1 per cent. phenol alone or 1 per cent. phenol and 60 per cent. glycerin both to the emulsion brings about a quicker elimination of bacteria, but this is accompanied by a more rapid deterioration of the virus itself. The same is true with the addition of one part per thousand of oil of cloves to 60 per cent. glycerin.

Recently Fornet (19), who has experimented with cultivation *in vitro* of vaccine virus, has recommended ether for removing the bacteria which do not possess spores. An emulsion of the virus can be freed of all bacteria except sporogenous ones by being shaken at room temperature for forty-eight hours with an excess of ether. The activity of the virus is, however, considerably diminished by ether treatment. By the ether method a vaccine pulp devoid of spore-bearing bacteria obtained as follows was secured free of all bacteria, and hence was suitable for cultivation in a pure state *in vivo*.

A sample<sup>2</sup> of glycerinated virus free from sporogenous bacteria was incubated at 37° C. for two days or longer, in order to destroy practically all the bacteria still present in it. The skin on the dorsal side of a rabbit was shaved and thoroughly cleansed with soap and rinsed with sterile distilled water. It was again shaved closely, after which the glycerinated virus was applied, in such strength as to produce separate eruptions. The vaccinated surface is protected from contamination by means of a sterile bandage. On the fourth or fifth day the bandage was removed and the surface washed first with absolute alcohol and then with ether. Several vesicles were selected and each was cleansed with 5 per cent. lysol solution and washed alternately with absolute alcohol and ether on sterile gauze. The eruptions were scraped with the edge of a sharp scalpel and the scrapings emulsified in several cubic centimeters of sterile saline solution. The emulsion was mixed with several volumes of ether and shaken in a sealed vessel for varying periods of time at room temperature. Samples were removed at the expiration of 1, 2, 4, 8, 12, 24, and 48 hours, from each of which cultures were prepared. When spores are absent sterility is usually obtained in twelve hours. The vaccinal activity falls at times to one-fifth of the original strength, but the characteristic properties remain unaltered.

#### TESTICULAR CULTIVATION IN RABBITS.

The vaccine emulsion so prepared is employed for the intratesticular inoculation of rabbits. Rabbits with well developed testicles should be chosen. After ether anesthesia an assistant holds the animal and fixes the testicles to prevent their withdrawal into the peritoneal cavity. The scrotal skin which is tightly stretched over the testicle is sterilized with 5 per cent. lysol solution and then painted with tincture of iodine. The operator next inserts the needle of a

<sup>2</sup> Several samples of the vaccine virus employed in the present investigation were furnished me by Dr. F. S. Fielder, Assistant Director of the Vaccine Laboratory of the Department of Health of the City of New York, to whom I wish to express my gratitude.

sterile syringe containing a 1 to 10 or 1 to 20 dilution of the emulsion into the testicular parenchyma along the long axis. The point of the needle is prevented from passing through the parenchyma to the tunica vaginalis. The contents are now gently forced out of the syringe into the organ at different regions by turning the direction of the needle. About one cubic centimeter of emulsion is injected into a testicle weighing from two to three grams. The organ is gently massaged to distribute the virus throughout the entire organ. The operation is practically painless.

The method just described is employed for the inoculation of the testicular strains of the virus, in which case an emulsion of testicle previously inoculated with the virus is used. The stock emulsion is prepared by grinding up the aseptically removed organs with sterile saline or 60 per cent. glycerin solution in the proportion of one gram of the tissue to two or three cubic centimeters of the fluid and any dilution of it; about 1 to 10 or 1 to 20 in saline is prepared for the purpose of the injection.

The testing for bacteria in the emulsion is an important point. Cultures are set up with plain and glucose bouillon, ascitic fluid with and without bouillon, and ascitic fluid with a piece of fresh sterile rabbit kidney with and without a layer of sterile paraffin oil. The cultures are incubated at 37° C. for three to four days, and then subcultures on plain and ascitic glucose slant agar, deep layer glucose agar, and ascitic tissue deep layer agar are made.<sup>3</sup> Film preparations stained by Gram or by Giemsa are also examined under the microscope, both from the cultures and from the testicles removed from the animal. In order to avoid occasional bacterial infection it has been found well to inoculate both testicles of each animal. The skin may be vaccinated at the same time. It is, moreover, advisable to use at least two rabbits for each transfer, because sometimes a rabbit reacts poorly to the vaccinal inoculation of both skin and testicle; but once the virulence of the virus has reached a certain height this precaution is no longer necessary.

<sup>3</sup> In the latter part of the present work initial culture in tissue bouillon and subcultures in glucose agar (deep) and plain agar (plate) were found to be sufficient.

## RESULTS OF EXPERIMENTS WITH RABBITS.

As the accompanying table shows, the passage of the testicular virus from animal to animal offered some difficulty until it had been carried on for a number of generations. In this experiment success was not assured until the virus had been transferred about ten times. That this result was due to increase in virulence of the virus in the later generations may be deduced from the greater activity manifested when it was tested upon the skin, as well as from the greater severity of the reactions in the testicles themselves (table I).

TABLE I.

Date of transfer.	Generation.	Results.	Date of transfer.	Generation.	Results.
1914					
Jan. 29-Feb. 2.....	1	+	Sept. 28-Oct. 3.....	32	++
Feb. 2-9.....	2	+	Oct. 3-7.....	33	++
Feb. 9-14.....	3	+	Oct. 7-12.....	34	++
Feb. 14-22.....	4	+	Oct. 12-16.....	35	++
Feb. 23-27.....	5	+ <sup>4</sup>	Oct. 16-20.....	36	++
Mar. 10-16.....	6	+ <sup>5</sup>	Oct. 20-25.....	37	+++
Apr. 22-25.....	7	+ <sup>6</sup>	Oct. 25-29.....	38	+++
May 1-6.....	8	+	Oct. 29-Nov. 3.....	39	+++
May 25-29.....	9	+	Nov. 3-7.....	40	+++
May 29-June 3.....	10	++	Nov. 7-12.....	41	+++
June 3-6.....	11	++	Nov. 12-16.....	42	+++
June 8-12.....	12	++	Nov. 16-21.....	43	+++
June 12-15.....	13	++ <sup>7</sup>	Nov. 21-26.....	44	+++
June 18-25.....	14	++	Nov. 26-30.....	45	+++
June 25-July 2.....	15	++	Nov. 30-Dec. 5.....	46	+++
July 2-8.....	16	++	Dec. 5-9.....	47	+++
July 8-13.....	17	++	Dec. 9-13.....	48	+++
July 13-16.....	18	++	Dec. 13-16.....	49	+++
July 17-20.....	19	++	Dec. 16-20.....	50	+++
July 20-23.....	20	++	Dec. 20-24.....	51	+++
July 26-30.....	21	++	Dec. 27-31.....	52	+++
July 30-Aug. 3.....	22	++	1915		
Aug. 4-7.....	23	++	Jan. 4-8.....	53	+++
Aug. 11-17.....	24	++	Jan. 8-12.....	54	+++
Aug. 17-22.....	25	++	Jan. 15-19.....	55	+++
Sept. 1-4.....	26	++	Jan. 23-27.....	56	+++
Sept. 7-10.....	27	++	Jan. 31-Feb. 4.....	57	+++
Sept. 12-16.....	28	++	Feb. 8-12.....	58	+++
Sept. 16-20.....	29	++	Feb. 16-20.....	59	+++
Sept. 21-24.....	30	++	Feb. 24-28.....	60	+++
Sept. 24-28.....	31	++			

<sup>4</sup> From this material two further generations were carried on and then lost, so it was necessary to come back to this generation and to start again.

<sup>5</sup> From this two generations were carried on and lost, necessitating a return.

<sup>6</sup> Only one out of several rabbits gave good results.

<sup>7</sup> Two rabbits were inoculated with this material, but only one gave a satisfactory result.



The testicular strain indicated in the table has passed through sixty successive generations within twelve months. It is interesting to find that considerable resistance to testicular adaptation was exhibited at the seventh transfer, after which no serious obstacle was met in carrying on the passages.

#### TESTICULAR VACCINAL PROCESSES IN RABBITS.

In order to follow the course of the vaccinal processes in the testicular tissue the following experiments were carried out.

Thirteen male rabbits were inoculated into each testicle with 1 c.c. of a 1:20 dilution of the saline testicular emulsion derived from the rabbit inoculated with the thirtieth generation of the testicular strain and castrated under ether anesthesia on the fourth day.

The testicles of these animals were removed one after the other and tested successively for activity on the skin, cornea, and testicles of normal rabbits every twenty-four hours, over a period of eighteen days, and then after 3, 4, 5, and 8 weeks.

Table II shows the results obtained.

During the first twenty-four hours the testicle presents little change, except that microscopic foci of infiltration of polynuclear leucocytes and exudate are observed in the interstitial spaces (figure 32, compare with normal, figures 30 and 31). At the end of forty-eight hours the swelling and induration of the organ begin to increase rapidly and the testicle becomes congested and edematous (figure 2). The content of the virus, which was almost zero after twenty-four hours, now reaches about 100 times that found at the end of the first day. When examined in sections an enormous leucocytic infiltration is seen in the interstitial tissues, and some leucocytes are contained in tubules (figure 33). The testicular cells are hydropic and fill up the tubular lumen. At the end of three days the infiltration has increased in intensity and extent (figures 3 and 18, compare with normal, figure 17), and the vaccinal activity has risen to at least 300 times that present twenty-four hours and about three times that present forty-eight hours after the inoculation.

The external changes present in the four day specimen (figures 4 and 34) resemble those occurring in the three day specimen, except that the testicle is more compact and less elastic and the amount of

TABLE II.

Testicles removed after	Gross appearance of specimens.	Tests for activity of emulsions on rabbits.			
		Dilution of emulsion.	Skin.	Cornea.	Testicle.
24 hrs.	Almost no swelling; traumatic hemorrhages	1 : 10	Eruption	—	+
		1 : 100	—	—	—
		1 : 1,000	—	—	—
48 hrs.	Vascular injection, edema, and moderate induration (figure 2)	1 : 10	++	++	++
		1 : 100	+	—	—
		1 : 1,000	<+	—	—
3 dys.	Marked induration, great congestion, greyish mottling, edema (figure 3)	1 : 10,000	—	—	—
		1 : 10	++	++	++
		1 : 100	++	+	—
4 dys.	Severe congestion, edema, induration and swelling; numerous yellowish grey spots on the organ (figure 4)	1 : 1,000	+	—	—
		1 : 10,000	—	—	+
		1 : 10	Confluent	++	++
5 dys.	Similar to the last (figure 1)	1 : 100	+++	+	—
		1 : 1,000	++	+	—
		1 : 10,000	<+	—	++
5 dys.	Similar to the last (figure 5)	1 : 10	Confluent	++	++
		1 : 100	Confluent	++	—
		1 : 1,000	++	+	—
5 dys.	Extensive hemorrhages; otherwise similar to the last	1 : 10,000	+	?	++
		1 : 10	Confluent	++	++
		1 : 100	++	+	—
6 dys.	Marked induration, but somewhat less than on the previous day. Less edema (figure 6)	1 : 1,000	+	—	—
		1 : 10,000	—	—	+
		1 : 10	Confluent	++	++
7 dys.	Induration somewhat less than on the preceding day. Yellowish grey specks prominent on the surface (figure 7)	1 : 100	++	+	—
		1 : 1,000	+	—	—
		1 : 10,000	—	—	+
8 dys.	General induration disappearing; organ flabby; disseminated yellowish grey spots (figure 8)	1 : 10	+	++	++
		1 : 100	<+	—	—
		1 : 1,000	—	—	—
9 dys.	Induration gone; soft and pale, slightly below original volume; some focal infiltration (figure 9)	1 : 10,000	—	—	—
		1 : 10	+	+	++
		1 : 100	<+	—	—
10 dys.	Induration gone; general atrophy; some focal induration (figure 10)	1 : 1,000	<+	—	—
		1 : 10,000	—	—	—
		1 : 10	+	+	++
11 dys.	Marked reduction in size; no induration (figure 11)	1 : 100	<+	—	—
		1 : 1,000	—	—	—
		1 : 10,000	—	—	—
12 dys.	No induration, soft and pale, much smaller than original (figure 12)	1 : 10	+	+	+
		1 : 100	—	—	—
		1 : 1,000	—	—	—
		1 : 10,000	—	—	—

TABLE II.—*Concluded.*

Testicles removed after	Gross appearance of specimens.	Tests for activity of emulsions on rabbits.			
		Dilution of emulsion.	Skin.	Cornea.	Testicle.
13 dys.	Normal in size, pale and soft; some greyish spots	1 : 2	+	+	.....
		1 : 10	< +	.....	+
		1 : 20	—	—	.....
		1 : 100	—	.....	.....
14 dys.	Similar to the last	1 : 2	—	—	.....
		1 : 10	—	.....	+
		1 : 20	—	.....	.....
15 dys.	Small, pale, flabby; few greyish foci	1 : 2	+	+	.....
		1 : 10	< +	—	+
		1 : 20	< +	—	.....
16 dys.	Similar to the last	1 : 2	+	+	.....
		1 : 10	< +	—	+
		1 : 20	< +	—	.....
17 dys.	Marked atrophy	1 : 2	—	—	+
		1 : 10	—	—	.....
		1 : 20	—	—	.....
18 dys.	Similar to the last	1 : 2	—	—	—
		1 : 10	—	—	—
		1 : 20	—	—	—
21 dys.	Small, fibrous; few minute foci of infiltration	1 : 2	< +	+	+
28 dys.	Similar to the last	1 : 10	—	—	.....
		1 : 2	1 eruption	—	+
35 dys.	Marked atrophy; small area of necrotic tissue present	1 : 10	—	—	.....
		1 : 2	—	—	—
		1 : 10	—	—	—
56 dys.	One testicle reduced to a small fibrous mass; the other less atrophied	1 : 2	—	—	—
		1 : 10	—	—	—

exudate in the tunica vaginalis and in the testicular tissue itself is more copious. The color of the testis has become purplish red, spotted here and there with irregular yellowish areas of different dimensions (figures 13 to 16). The organ is easily torn. The sections show numerous groups of several tubules each, which have lost the property of taking up the basic stain, indicating a total necrosis of the structures (figures 19 and 36). These changes are not universal, since areas exist in which no apparent serious alteration has taken place. At this stage the vaccinal power is nearly three times as great as it was at the end of three days, and at least 1,000 times as great as at the end of the twenty-four hour period. The changes in the five day specimen are about the same as those of the four day specimen. The vaccinal activity seems now to have attained its maximum height, since almost confluent eruptions on the skin of rabbits are yielded in dilutions of 1 to 1,000. The sections indicate beginning disintegration of the leucocytic elements and of

affected tubular cells (figures 5, 29, 29 a, and 35; compare with normal, figure 28).

At the expiration of six days the testicle has become of softer consistency (figure 6), and the edema and cellular infiltration have begun to recede. Microscopically foci of small round cells in the infiltrated areas and fibrin masses are distinguished (figures 37 and 38). The vaccinal activity is similar to that of the five day specimen. After seven days the resolution of the infiltration proceeds more rapidly (figures 7 and 20), while the vaccinal activity is less than that of the preceding day. From now on the testicles diminish rapidly in volume, so that the ten day specimen is below normal in size (figures 8, 9, 10, 21, and 22). The organs are now pale and of soft texture. The activity of the virus is much less than it was at the end of seven days. At the end of eleven days it is still weaker. From twelve to eighteen days the shrinkage of the organ continues and the sections show loss of testicular cells (figures 11, 12, 23 to 27, and 39 to 44) and collapse of the tubules. The virus has now disappeared wholly or almost wholly, and in some instances no vaccinal effect could be obtained in any concentration. Hence this period may be regarded as that of elimination of the virus, while the exact moment of disappearance probably depends upon the quantity of the virus originally developed and the degree of immunity displayed. At later periods, namely after five weeks, no virus could be detected, while the atrophy of the testicular parenchyma may be complete, so that a fibrous mass containing a few unusually thin tubules, lined with a single layer of epithelial cells may alone remain (figures 27 and 45).

The tunica vaginalis is often intensely infiltrated with polynuclear leucocytes (figure 46) and contains a considerable amount of the virus; apparently the virus multiplies here as it does in other epithelial cells. The epididymis shows a slight infiltration only during the early period.

Spermatogenesis ceases quickly and the various sperm cells quickly degenerate under the influence of the vaccinal process.

EFFECTS OF THE TESTICULAR VACCINAL STRAIN ON THE SKIN  
AND CORNEA.

The testicular strains of the virus were employed to inoculate the shaved skin and scarified corneal surfaces of rabbits. The effects of the vaccinations so carried out may be followed in table III. They will be recognized as characteristic of the effects produced by active vaccine virus as usually prepared in the calf. The microscopical features of the process are also typical, including the presence of the Guarnieri, or vaccine, bodies so called (figures 49 and 50).

TABLE III.

At the end of	Average course in rabbits.		
	Skin.	Cornea.	Temperature. <sup>8</sup>
24 hrs.	Diffuse reddening	Slight swelling	38.9°
48 hrs.	Mottled reddish areas	Slight turbidity	39.5°
3 dys.	Fairly defined flat erythema, in part confluent	Distinct turbidity	40.0°
4 dys.	In part confluent, mostly discrete raised papules with induration and areola	Advancing in degree and extent	40.8°
5 dys.	Distinct raised round vesicles with areola	Sometimes ulceration	40.2°
6 dys.	Development into pustular eruptions	Ulceration	39.8°
7 dys.	Pustules with scanty content; tendency to dry up	No change	38.8°
8 dys.	Inflammatory processes disappearing	Ulcer persists	38.9°
9 dys.	Stage of crust formation and desquamation	Ulcer persists	38.7°
10 dys.	Stage of crust formation and desquamation	Ulcer persists	38.8°

EFFECTS OF VARYING CONCENTRATION OF TESTICULAR VIRUS UPON  
THE SKIN, CORNEA, AND TESTICLE.

In order to determine the sensitiveness of the skin, cornea, and testicle to vaccine virus, emulsions of the testicular virus of different concentrations were prepared and inoculated into the several parts mentioned.

For this purpose a specimen of testicle representing the thirty-first passage or generation was employed. The dilutions in sterile saline were as follows: 1 to 10, 1 to 100, 1 to 300, 1 to 1,000, 1 to 3,000, 1 to 10,000, 1 to 30,000, 1 to 100,000. The skin and cornea were

<sup>8</sup> Average from fifteen rabbits.

inoculated in the usual manner, and 0.5 of a cubic centimeter was injected into the testicle.

The result is summarized in table IV and may be stated as follows: Up to a dilution of 1 to 300 the testicular emulsion causes marked vaccinal reactions in the skin, cornea, and testis. The skin surface still reacts slightly at the 1 to 1,000 dilution, but the cornea does not. Beyond the 1 to 1,000 dilution the skin reacts no longer, while the testicle continues to react even to dilutions of 1 to 100,000, the limit of the experiment. However, dilutions of 1 to 3,000 and higher retarded somewhat the testicular reaction, which in the end

TABLE IV.

Dilution of emulsion.	Reactions following the inoculation of different dilutions of the emulsion as indicated in the first column.			Tests of activity of emulsions prepared from testicles <sup>9</sup> which had been injected with different dilutions.			
	Skin.	Cornea.	Testicle.	Dilution of emulsion of each testicle.	Skin.	Cornea.	Testicle.
1 : 10	+++	+	3 dys. already marked induration and edema	1 : 100	+	+	++
1 : 100	++	+	3 dys. already marked induration and edema	1 : 1,000	+	.....	++
1 : 300	++	+	3 dys. already marked induration and edema	1 : 100	++	++	++
1 : 1,000	+	—	3 dys. already marked induration and edema	1 : 1,000	+	.....	++
1 : 3,000	—	—	3 dys. only slight induration; 5 dys. marked increase	1 : 100	++	++	++
1 : 10,000	—	—	3 dys. only slight induration; 5 dys. marked increase	1 : 1,000	+	.....	++
1 : 30,000	—	—	3 dys. only slight induration; 5 dys. marked increase	1 : 100	++	++	++
1 : 100,000	—	—	3 dys. only slight induration; 5 dys. marked increase	1 : 1,000	+	.....	++

was quite identical with that produced by the stronger concentration. In an experiment made somewhat later and after the testicular strain of the virus had been intensified by repeated passages, a skin reaction of small degree but of characteristic kind could be elicited in the 1 to 100,000 dilution.

This experiment serves also to bring out distinctly the fact of

<sup>9</sup> The testicles were removed from the animals on the sixth day after the inoculation.

the rapid multiplication of the virus in the testicle of rabbits, and is shown in the second half of table IV. The vaccinal effect upon the skin, cornea, and testicle became essentially the same in kind and degree, irrespective of the concentration of the virus emulsion injected originally into the testicle. At the expiration of six days the testicles inoculated with virus in the 1 to 100,000 dilution yielded a virus emulsion as active as the original testicular material employed for preparing the stock emulsion. The rapidity and ease of multiplication of the virus within the testicle is well illustrated by this experiment. The causes of the small variations in effect as indicated in the table as occurring between emulsions of testicles receiving the higher and lower dilutions of virus are not at once apparent. Possibly certain rabbits restrain the multiplication of the virus more than others; possibly concomitant immunity reactions come into play.

#### TESTICULAR CULTIVATION IN BULLS.

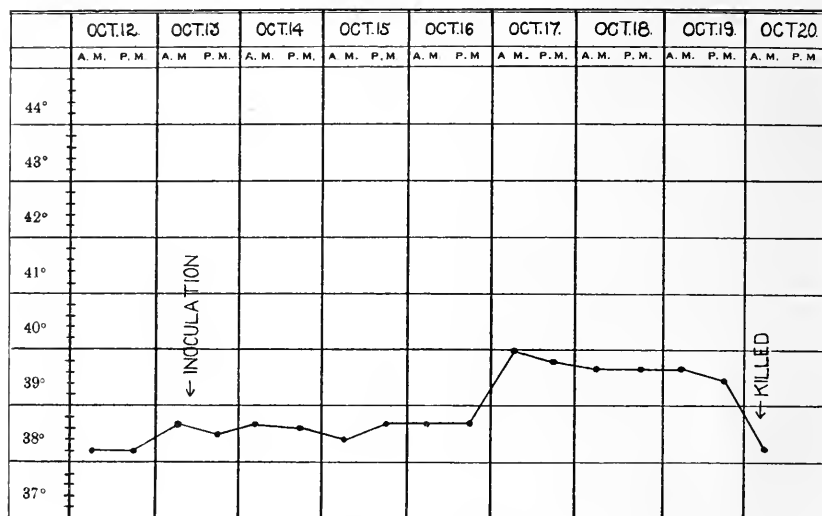
Experiments were next performed on the testicles of young bulls, with the view of ascertaining whether this species of animal would be suitable for use, both from the quantitative and qualitative relation of the virus, since it has long been customary to produce vaccine in the skin of this species.

*Bull 1.*—Weight, 750 pounds. The scrotal skin was shaved, washed with soap, and sterilized by sublimate alcohol. The vaccine virus employed for the injection consisted of testicular emulsion derived from the testicles of two rabbits which had been inoculated with the 30th generation of the testicular strain and castrated at the end of seven days. This emulsion produced a fairly thick eruption on the skin of rabbits in the dilution of 1:100. On Oct. 13, 1914, 10 c.c. of the emulsion diluted 1:100 were injected into the right, and 15 c.c. into the left testicle under aseptic precautions.

The testicles showed signs of swelling on the third day (forty-eight hours), and increased further in size and density up to the end of six days. The left testicle was much more swollen than the right. The temperature remained normal ( $38.7^{\circ}$  to  $38.9^{\circ}$ ) until the end of the fifth day, when it rose to  $40.6^{\circ}$ ,  $40.1^{\circ}$  (6th day), and remained at  $40.4^{\circ}$  as late as the end of the sixth day. On the morning of the eighth day the temperature was  $38.7^{\circ}$  (text-figure 1).

The animal was slaughtered and the testicles were removed under aseptic precautions on the eighth day. The subcutaneous tissue and the tunica vaginalis were edematous and hemorrhagic, especially along the needle track. The testicles themselves were also edematous and showed a mass of coagulated blood at the site where a blood vessel must have been injured by the needle puncture.

Compared with the normal the vaccinated testicles were firmer and more pinkish in color, besides showing numerous ecchymoses. The left testicle was more altered than the right; the former weighed 310 and the latter 250 gm.



TEXT-FIG. I. Bull I.

Emulsions in sterile saline solution were made with the tissues immediately about the point of insertion of the needle and also of portions remote from the site of injection. The tests for activity were made upon the shaved skin, cornea, and testicles of rabbits.

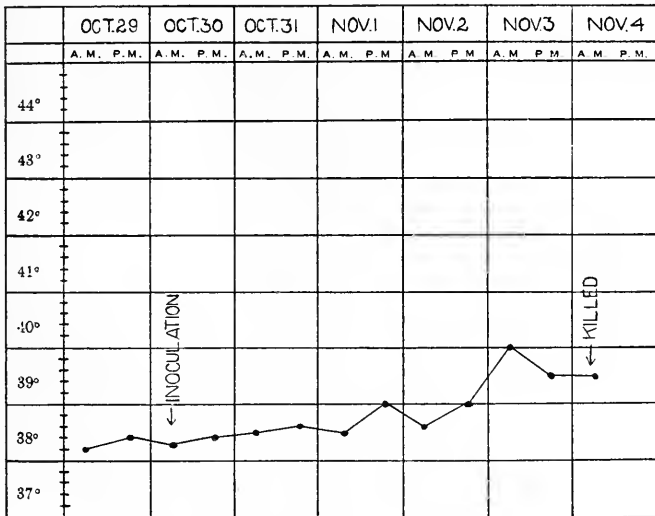
TABLE V.

Bull I.	Tests on rabbits.			
	Dilution of emulsion.	Skin.	Cornea.	Testicle.
Emulsion of tissue around site of injection. . . .	1 : 10	+++	++	++
	1 : 100	+	+	++
	1 : 1,000	<+	—	++
	1 : 10,000	—	.....	++
Emulsion of tissue remote from site of injection.	1 : 10	++	++	++
	1 : 100	+	—	++
	1 : 1,000	—	.....	—
	1 : 10,000	—	.....	—
Exudate in tunica vaginalis. . . . .	Undiluted	—	—	—
	1 : 10	—	—	—



The exudate contained in the tunica vaginalis was likewise tested. Both exudate and emulsions were found sterile for bacteria. Table V summarizes the results, which show that the virus multiplied to a much smaller extent in the testicular tissues of the bull than is the rule in the testicle of the rabbit. That the virus multiplied in some degree is highly probable; and that the edematous fluid in the tunica vaginalis was devoid of virus is an interesting point.

*Bull 2.*—Weight, 680 pounds. Oct. 30, 1914. The left testicle was injected at four different sites in order to distribute the virus widely with a 1:100 dilution of rabbit testicular virus in the thirty-third generation, the total quantity inoculated amounting to 20 c.c. At the same time the skin of the ventral surface below the navel was shaved, scarified, and inoculated with some of the same emulsion. The inoculated testicle after forty-eight hours was tense on palpation, distinctly swollen, and indurated. The swelling and induration increased during the next three days. The skin showed distinct papules three days after the inoculation, which became typical pustules two days later (figure 53). The temperature rose to 40° to 40.7° on the fourth day, and was 40° on the sixth day. The bull was slaughtered on the sixth day and the testicles were aseptically removed (text-figure 2).



TEXT-FIG. 2. Bull 2.

The scrotum was found to be edematous and infiltrated, but no adhesion was present between the tunica and the testicular surface. The moderate amount of serous and hemorrhagic exudate proved to be sterile for bacteria. Upon section

serous exudate oozed freely from the testicle. The points of injection could be detected by the presence of numerous minute hemorrhages within areas which were distinctly firmer than the surrounding parts. Coagula of blood occurred here and there (figures 54 and 55). The uninoculated right testicle showed no change. The weight of the left testicle was 229 and that of the right 199 gm.

Emulsions were prepared both from the tissues immediately about the sites of injection and from parts remote from them. The tests were made on the skin, cornea, and testicles of rabbits.

TABLE VI.

Bull 2.	Tests on rabbits.			
	Dilution of emulsion.	Skin.	Cornea.	Testicle.
Emulsion of tissue around zone of injection.	1 : 10	Confluent	++	++
	1 : 100	++	+	++
	1 : 1,000	+	.....	++
	1 : 10,000	< +	.....	++
Emulsion of tissue remote from site of injection.....	1 : 10	++	++	++
	1 : 100	+	+	++
	1 : 1,000	< +	.....	+
	1 : 10,000	-	.....	-
Exudatè in tunica vaginalis .....	Undiluted	< +	+	+
	1 : 30	-	-	-

Table VI, which summarizes the results, indicates that the tissues about the sites of inoculation contain an amount of the virus about equal to that of rabbit's testicles inoculated with similar material, and that the degree of activity possessed by the two testicular strains is about the same. On the other hand, the emulsion made from the remote portions of the testicle produced far less effect, although it was still active up to the dilution of at least 1 to 1,000. The exudate in the tunica contained a trace of the virus, but this may have been due to a contamination of this exudate in the opening of the testicle.

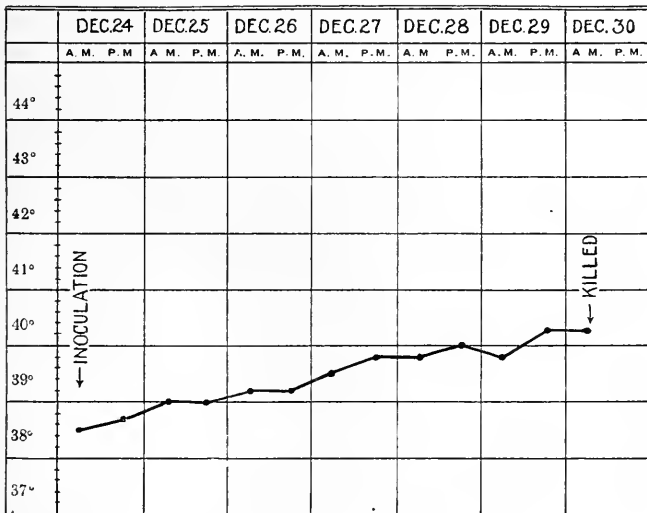
*Bull 3.*—Weight, 720 pounds. Nov. 11, 1914. Both testicles were inoculated with an emulsion of a testicular strain obtained from a rabbit inoculated with the testicular strain of bull 2. The emulsion was, however, contaminated with a *xerosis* bacillus. The local and general reactions were similar to those described in the previous two bulls. In spite of the admixture of *xerosis* bacilli with the virus the local reaction was not different from those following the injection of a pure virus. The animal was slaughtered on the ninth day after injection.

The testicular tissues were edematous, showed several well localized areas of

a greyish white color, and some hemorrhages caused by the trauma of the needle. The tissues were not softened. Cultures made from the emulsions of the testicles showed the presence of a few *xerosis* bacilli, but no other bacteria.

The vaccinal activity as tested on the rabbit showed that it was much weaker than that of bull 2.

*Bull 4.*—Weight, 140 pounds. Dec. 24, 1914. 4 c.c. of a 1:40 dilution of an emulsion of the fiftieth generation of testicular rabbit virus were injected into the left, and 3 c.c. of a 1:10 dilution of the testicular virus from bull 2 were injected into the right testicle. The organs were examined every twenty-four hours, but no particular difference was noticed on the two sides. Both showed marked induration within thirty hours. On the fifth day the swelling appeared to have reached the maximal stage. The temperature rose after three days and reached 40.8° on the sixth day. The animal was slaughtered at the end of six days (text-figure 3).



TEXT-FIG. 3. Bull 4.

The testicles had increased in volume, especially the one on the left side injected with the bull-rabbit strain; they were markedly edematous and congested. On section numerous greyish yellow specks were seen to be scattered throughout the organ, and were especially numerous along the sites of injection. Similar greyish yellow areas, it will be recalled, occur almost constantly in the inoculated rabbit's testicles.

The emulsions were made from both testicles separately and tested for their vaccinal activity, together with the controls of the standard glycerinated vaccine issued by the Department of Health of New

York City. The testicular emulsions were free of bacteria; the Health Department vaccine, of course, was not.

TABLE VII.

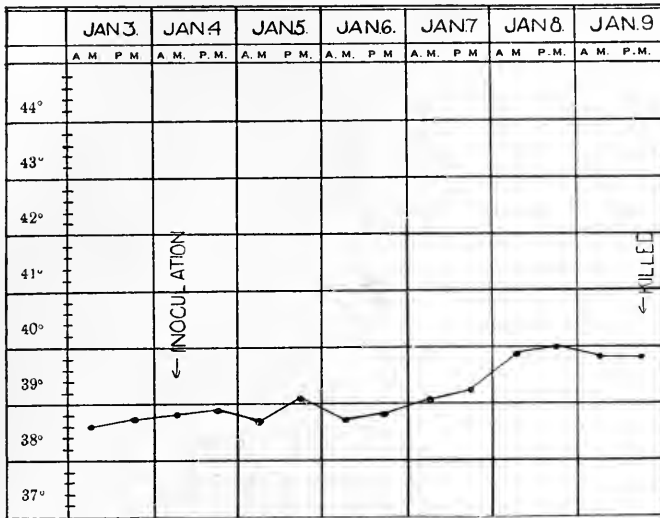
Bull 4.	Dilution of emulsion.	Tests on rabbits.			Tests on calves.	
		Skin.	Cornea.	Testicle.	Skin.	
Emulsion of testicle inoculated with bull-rabbit testicular strain	1 : 10	Confluent	++	++	Confluent.	
	1 : 100	Confluent	+	++	++	
	1 : 1,000	+	±	++	+	
	1 : 10,000	< +	-	++	< +	
Emulsion of testicle inoculated with bull-rabbit testicular strain no. 2	1 : 10	Confluent	++	++	Confluent.	
	1 : 100	Confluent	++	++	Almost confluent.	
	1 : 1,000	++	+	++	++	
	1 : 10,000	+	-	++	< +	
Regular vaccine as controls	N. Y. D. H. standard vaccine 1st sample	1 : 10	Confluent	++	++	Confluent.
		1 : 100	Confluent	++	++	Almost confluent.
		1 : 1,000	++	+	+	++
		1 : 10,000	< +	-	< < +	< +
	N. Y. D. H. standard vaccine 2d sample	1 : 10	Confluent	++	++	Confluent.
		1 : 100	++	+	+	++
		1 : 1,000	+	-	-	+
		1 : 10,000	-	-	-	-

The above experiments (table VII) demonstrate that a strong bacteria-free vaccine virus can be produced in the testicular tissues of the young bull by injecting the testicular strain derived from a bull or from a rabbit, and they also show that the vaccinal activity reaches the same strength as that possessed by a standard virus propagated on the skin of a calf. Moreover, no differences were noted in the type and course of the vaccinal processes as produced in the skin of rabbits and calves by the vaccines from the several sources. The only distinctions are quantitative ones, since the skin virus is more quickly diluted beyond effective strength than the testicular virus.

The next experiment was devised to compare the effect of the testicular inoculation of a testicular and a skin strain of vaccine, respectively, into the same calf.

*Bull 5.*—Weight, 160 pounds. Jan. 4, 1915. 3 c.c. of a 1 : 10 dilution of an emulsion of the testicular strain derived from bull 4 were injected into the right, and 3 c.c. of a 1 : 10 dilution of the New York City Department of Health standard vaccine into the left testicle. The next day the swelling and injection of the right testicle were marked, while the left was far less altered. This difference

became more pronounced, and on the sixth day only the right side presented the typical reaction, swelling, and induration previously noted, while the left was but slightly enlarged. The animal was slaughtered at the end of five and one-half days (text-figure 4).



TEXT-FIG. 4. Bull 5.

Upon exposing the testicles it was found that the left showed infiltration in small amount and only about the site of the injection, while the right was edematous throughout, greatly enlarged, and dotted with numerous minute foci of greyish yellow appearance. While the consistency of the left testicle which weighed 6.5 gm. was about normal, that of the right, which weighed 8 gm., was diminished.

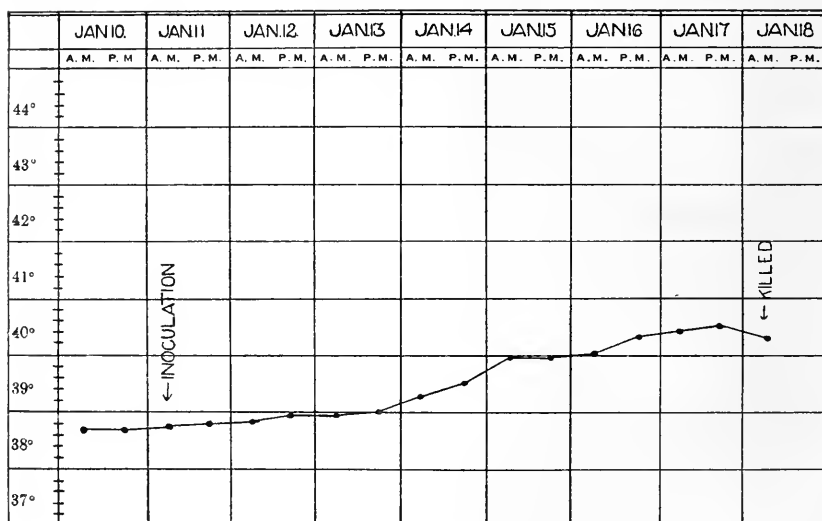
The vaccinal strength of both testicles was tested on the rabbit.

TABLE VIII.

Bull 5.	Tests on rabbits			
	Dilution of emulsion.	Skin.	Cornea.	Testicle.
Emulsion of testicle inoculated with calf testicular strain no. 4	1 : 10	Confluent	++	++
	1 : 100	++	++	++
	1 : 1,000	+	-	++
	1 : 10,000	-	-	++
Emulsion of testicle inoculated with skin strain (N. Y. D. H.)	1 : 10	+	+	+
	1 : 100	< +	-	-
	1 : 1,000	-	.....	-
	1 : 10,000	-	.....	-

As table VIII indicates, the local reaction and the vaccinal activity were more marked in the case of the testicular than of the skin strain, from which it may be inferred that adaptation of the strain to the testicle is necessary as a prerequisite to the production by this means of strong vaccine.

*Bull 6.*—Weight, 225 pounds. Jan. 11, 1915. The left testicle was injected with an emulsion of the testicle that had been inoculated with the standard skin strain of vaccine, as mentioned above, and the right testicle with an emulsion of the testicle previously inoculated with a testicular strain. The local and general reactions were similar to those observed and described, except that the left testicle, weighing 9 gm., showed far less change than the right, which weighed 12.5 gm.



TEXT-FIG. 5. Bull 6.

(figures 56 and 57). The bull was slaughtered at the end of five and one-half days, and the vaccinal activity of each testicle was tested on the rabbit (table IX and text-figure 5).

*Histological Changes.*—The histological changes observed in the testicles of bulls inoculated with testicular strains of vaccine virus are similar to those enumerated in these organs of the rabbit. An acute inflammatory reaction arises, in which many polymorphonuclear leucocytes invade the interstitial tissues together with a serous exudate; the adjacent tubules are compressed by pressure of the exu-

TABLE IX.

Bull 6.	Tests on rabbits.		
	Dilution of emulsion.	Skin.	Cornea.
Emulsion of left testicle inoculated with skin strain.	1 : 10	+	+
	1 : 100	< +	-
	1 : 1,000	-	.....
	1 : 10,000	-	.....
Emulsion of right testicle inoculated with testicular strain	1 : 10	+++	++
	1 : 100	++	+
	1 : 1,000	+	.....
	1 : 10,000	-	.....

date, which also invades them, and the epithelial cells degenerate. The reaction is multifocal and affects parts remote from the site of inoculation, although the severer effects occur adjacent to the inoculation. Hemorrhages occur also (figures 57 to 66).

#### IDENTITY OF THE SKIN AND TESTICULAR STRAINS OF VACCINE VIRUS.

It may now be considered as established that vaccine virus derived from the specific skin lesions is capable of multiplying indefinitely in a pure state within the testis of the rabbit and the bull. There would appear to be no necessity for restoring the virus to skin surfaces in order to continue the propagation in the testicles. The testicular epithelium, once the virus is adapted to it, seems to provide as suitable a medium for its multiplication as the skin epithelium. Moreover, in order that the virus should propagate in the testis no previous injury of the organ is necessary, save that caused by the injection, so that in this respect the testicle may be regarded as exceeding the skin surface in susceptibility to its presence. Possibly anatomical structure accounts for this difference; unless scarified the virus has no opportunity of adhering to and entering intimately into relation with the skin surface, while injection into the testicle brings the virus at once into immediate proximity to the tissues composing that organ.

The question which arises is whether the skin and testicular strain of virus remain identical in physiological properties. One has, of course, been derived from the other; but it remains to be considered whether in the course of adaptation the testicular strain has undergone modification.

Several criteria can be employed to decide this point: (a) type and course of the eruption of the skin of susceptible hosts (rabbit, calf, man); (b) characteristic lesion of the cornea of the rabbit; (c) histological processes within the lesions caused by the virus; (d) phenomena of immunity arising after vaccination; (e) resistance to the action of certain agencies, such as glycerin, ether, chloroform, phenol, drying, and heating.

It may be assumed that merely completely freeing vaccine virus from the usual bacteria which contaminate it in practice will not change its physiological properties. This probability is indicated by the effect of glycerination which reduces the bacterial content. The practical advantage of this reduction is obtained in milder vaccinal effects in man, which are equally protective with the severer effects resulting from bacterial coöperation.

Hence it may be hoped that the complete removal of the contaminating bacteria should still further improve the results of vaccination in that the secondary operation of bacteria contained within the virus will be entirely eliminated. The employment of such a pure strain virus will, at the same time, do away entirely with the potential danger of contamination of the virus with tetanus bacilli or spores, a subject which has always engaged the attention of producers, and respecting which special precautions are taken.

*Skin Reactions.*—Both skin and testicular strains of virus were inoculated simultaneously on different parts of the shaved skin surfaces of rabbits and calves, after which the course of the vaccination was followed. No differences were noted (figures 67, 68, and 69).

*Cornea.*—The rabbit's cornea inoculated with skin and testicular virus, respectively, undergoes the same series of changes, and in each case Guarnieri bodies are produced. The results are indistinguishable (figures 49 and 51).

*Histological Lesions.*—When the testicular strains of virus are reimplanted upon the skin of the rabbit and calf, and the minute histological changes produced are compared with those caused by the skin strains of virus, no essential differences have been detected (figures 47, 48, 50, and 52). Moreover, the microscopical changes in the testicles produced by skin or adapted testicular strains of virus are essentially identical.



## IMMUNITY PHENOMENA.

Considered from the practical standpoint the most important effect of vaccine virus is the immunity to reinoculation that it confers. Hence, experiments were conducted with the testicular strains in order to determine the immunity effects which it produces.

*Experiment I.*—Rabbit. First vaccination, Oct. 5, 1914, at three separate places on the shaved skin, with regular, glycerinated stock virus. Confluent vaccinal eruptions were produced.

Second vaccination, Feb. 12, 1915. The three places previously vaccinated were now revaccinated as follows: (a) an area with some of the virus used previously; (b) an area with rabbit testicular strain; (c) an area with bull testicular strain. The three areas reacted in identical manner: each area became congested, but neither vesicles nor papules appeared. The reddening of the skin was probably allergic in origin (20).

The control rabbit developed confluent eruptions from each of the three samples of virus.

*Experiment II.*—Rabbit. First vaccination, Oct. 5, 1914, at three separate places on the shaved skin, with a testicular strain of vaccine virus from a rabbit. Confluent vaccinal eruptions were produced.

Second vaccination, Feb. 12, 1915. The three places previously vaccinated were revaccinated as follows: (a) an area with some of the virus used previously; (b) an area with bull testicular strain; (c) an area with New York City Department of Health vaccine. On the following day slight transient scattered erythema was noted, but neither papules nor pustules.

The control rabbit developed typical confluent eruptions from each of the three samples of virus.

*Experiment III.*—Rabbit. First vaccination, Feb. 2, 1915, at three places on the shaved skin, with testicular strain from a bull calf (No. 4). Confluent vaccinal eruptions were produced.

Second vaccination, Feb. 17, 1915. The three places previously vaccinated were revaccinated as follows: (a) an area with some of the virus used in the first vaccination; (b) an area with a rabbit testicular strain; (c) an area with the regular New York City Department of Health vaccine. Only moderate allergic reactions were observed.

Two other rabbits similarly tested gave identical results with the above experiment, except for the presence in one of them of a slight erythema along the scarified lines, which lasted three days.

Controls on a normal rabbit showed that the strength of the vaccine strains employed for the foregoing experiments was such as to produce confluent eruptions.

The experiments described are conclusive in demonstrating that the skin and testicular strains of vaccine virus yield identical immunity reactions both as regards kind and degree.

TESTICULAR CULTIVATION AS A MEANS OF PROPAGATION OF  
PURE VACCINE VIRUS.

It is obvious that a method of propagating the vaccine virus free from bacterial contamination would remove all danger of accidental infection through the introduction at the time of vaccination of an impure preparation containing certain bacteria. Without stopping to consider how often severe effects have resulted from bacterial contamination of the virus it may be urged that the use of a pure preparation will altogether preclude the possibility of a mixed infection from this source. The indefinite propagation of a highly potent vaccine virus in the testicular tissues of animals is comparatively a simple procedure, so that by means of a strictly aseptic technique combined with careful bacteriological control of the product, it will now become a simple matter to supply the medical profession with an absolutely pure virus, for the purpose of vaccination. As is well known, the method hitherto practised for propagating vaccine consists in utilizing the emulsion of the skin pulp from vaccinated calves. The usual fresh pulp contains many and various bacteria, so that to free the pulp of them it must be left in contact for a period with 60 per cent. glycerin or 1 per cent. phenol, before it is regarded as ready for use on human subjects. Since the strength of the virus also deteriorates with time the process of vaccine preparation has not been perfected from the practical point of view. On the other hand, a pure virus should be ready for use as soon as the usual bacteriological and potency tests are completed, that is, within about one week after the preparation of the emulsion of the testicle containing it.

The introduction of a pure vaccine into the public health service would be welcome, even if it were somewhat more expensive to produce than the usual vaccine. As a matter of fact it appears to be even more economical than the latter, as the following calculation shows.

A male rabbit weighing about 3 kg. yields from 7 to 10 gm. of testicular material containing the pure culture of vaccine. When this amount of tissue is emulsified in from 15 to 20 c.c. of 50 per cent. sterile glycerin, it will yield from 20 to 28 c.c. of a finished product of high potency. A sample of this product is diluted 1:1,000 and tested on the skin of rabbits. If a confluent eruption results,

which may reasonably be expected, the stock suspension may be further diluted with four times its volume of sterile 50 per cent. glycerin, since a sample of glycerinated virus which produces a confluent eruption in a dilution of 1:200 is sufficiently strong for use in man. Hence a single rabbit may yield 125 c.c. of finished glycerinated virus, or even more.

The average yield from the calf, according to Fielder,<sup>10</sup> is put at 40 to 100 gm. of fresh pulp, which emulsified with four parts of 50 per cent. glycerin gives 250 to 300 c.c. of vaccine virus ready for use within three months.

A comparison of the cost is in favor of the rabbit vaccine. For the preparation of 1,000 cubic centimeters of the finished vaccine product about 40 rabbits or 4 calves would be required. The initial cost of the former would be considerably smaller.

Moreover, the employment of rabbits instead of calves has advantages of another kind from those which have just been emphasized. The prevalence of foot-and-mouth disease among cattle has closed the market in many states to calves, so that the propagation of vaccine virus in the ordinary way has recently suffered. No such interruption is to be feared with respect to the use of rabbits. During the prevalence of such an epidemic it becomes essential also to keep the calves intended for vaccination under observation before use for a sufficient period to insure their freedom from foot-and-mouth disease infection.

Finally, it may be added that the bull may also come to be used for the testicular cultivation of the virus. However, the method has not yet been worked out for this animal as completely as it has for the rabbit. Hence it must be left to the future to determine whether the bull is strictly suitable for the purpose. This part of my work suffered interruption because of the prevailing epidemic of foot-and-mouth disease and the resulting quarantine upon cattle.

#### STANDARDIZATION OF VACCINE VIRUS.

Several methods are employed for determining the efficacy and potency of vaccine virus before distributing it for general use. The accepted methods agree in using animals before permitting the virus to be used on man. Theobald Smith recommends the skin of the calf, and requires that the eruptions produced there must correspond with those of the skin of a child. Calmette and Guérin (9, 21) and

<sup>10</sup> On the other hand, Rosenau (1) gives the amount of fresh pulp from a calf as 20 to 40 gm., which will yield 50 to 120 c.c. of the glycerinated emulsion.

Camus (22) employ the skin of the rabbit; while Chaumier (23) and others<sup>11</sup> first employ animals, and then themselves observe the vaccination effects on a few children before issuing the virus. There is agreement among the authorities that the skin of the rabbit and calf are reliable indications of the effects produced on human subjects.

Henseval and Convent (24) consider that a preparation which produces on the skin of the rabbit almost confluent eruptions in a dilution of 1 to 500 should be considered a suitable vaccine. Such a sample always causes an uninterrupted eruption along a line (linear eruption) on the skin of a child. They recommend as still usable even a weaker specimen provided it produces a continuous eruption in a 1 to 100 dilution.

The testicular vaccine strains which the author prepared in rabbits either fulfill or exceed the above requirement, since a few samples produced confluent eruptions in dilutions of 1 to 10,000. On the other hand, the products from the testicles of the bull were less active, and a few only of the preparations reached the lower standard.

#### PURE VACCINATION IN MAN.

The tests which were carried out in detail and with minute care in animals with the pure testicular strains of vaccine virus all indicated that the virus would produce typical vaccinal effects in the human subject.

Several adults, chiefly physicians familiar with the cultivation experiments, immediately volunteered for vaccination, which was carried out by the linear and scarification methods. Among the tests, which were all revaccinations, some were positive and others negative. In the positive results the vaccinal eruption was typical and no secondary infection arose to complicate or intensify the vaccination.

Among those in whom the vaccination was positive was a physician. After his experience he vaccinated his own child, three years and two months old, who had not previously been successfully

<sup>11</sup> The Department of Health of the City of New York issues only the preparations which have produced on primary vaccination cases fifteen typical takes on fifteen insertions made on five children, thus requiring 100 per cent. of positive takes for each sample of vaccine.

vaccinated. The linear eruption appeared on the fifth day, increased in intensity during the three succeeding days when it reached the maximum, and then receded. No pain or itching was complained of, and the highest temperature was  $37.5^{\circ}$  C., which was noted on the eighth day. On the fourteenth day scab formation was well advanced. I am indebted to the father of the child for the two photographs (figures 70 and 71) which illustrate the vaccinal effects on the fifth and sixth days.

#### SUMMARY.

Vaccine virus freed from all associated bacteria by means of suitable disinfecting agents can be propagated in a pure state in the testicles of rabbits and bulls. The virus cultivated in this manner is not only devoid of all bacteria, but appears capable of indefinite transfer from one animal to another. Sixty passages in rabbits of a pure strain have been made within one year.

Several transfers from testicle to testicle are required to bring about accurate adaptation of the virus to the testicular parenchyma, so that continued propagation in this way can be certainly secured. During the first transfers from testicle to testicle the activity of the virus may be less than the original skin specimen from which the pure strain was derived; but as the transfers proceed the activity rises until, when the adaptation is complete, the activity of the testicular equals that of the skin strain.

The multiplication of the virus within the testicle is maximum on the fourth or fifth day after inoculation; the quantity of virus remains about stationary until the eighth day, when diminution begins. At the expiration of five weeks no more virus could be detected in the testicle.

The vaccinal processes in the skin, cornea, and testicle of rabbits are practically identical whether the virus employed for the inoculation has been the original skin strain or the pure testicular strain; and the skin lesions produced in the calf with the two strains are also identical.

In conformity with the finding mentioned in the last paragraph it has been found that human beings react to the pure testicular strain of vaccine virus in an entirely typical manner. In the case

both of original vaccination and revaccination the vaccinal effects cannot be distinguished from those arising from uncomplicated skin virus.

Pure strains of testicular virus are readily produced, and once secured they may be propagated in a pure state by the method described in rabbits or bulls without difficulty and with economy. The pure strains thus obtained should supply an ideal form of virus for employment in the vaccination of human beings.

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## EXPLANATION OF PLATES.

## PLATE 39.

FIG. 1. The right testicle shows swelling due to the injection of pure vaccine virus made five days previously; the left side is normal.

FIGS. 2 to 12. The conditions of the testicles at various stages in the vaccinal processes produced by means of a pure vaccine virus, as follows: 2, 48 hours; 3, 3 days; 4, 4 days; 5, 5 days; 6, 6 days; 7, 7 days; 8, 8 days; 9, 9 days; 10, 10 days; 11, 11 days; and 12, 12 days after the inoculation. The yellowish grey mottles of irregular dimension can be seen in the specimens shown in figures 4, 5, and 6. The marked atrophy of the organ is easily noticeable in the specimens shown in figures 9, 11, and 12. All natural size.

## PLATE 40.

FIG. 13. A testicle four days after inoculation with pure vaccine virus. It shows marked vascular injection, edema, and several small yellowish grey spots visible on the surface.

FIG. 14. The organ on section, in which several lobules can be seen undergoing necrosis, as indicated by the greyish yellow, somewhat indurated areas. The parenchyma has bulged out of the tunica albuginea on account of the edema.

FIGS. 15 and 16. Two specimens of testicles at the height of the vaccinal processes, one (figure 16) with hemorrhagic reactions. In the latter instance the necrotic changes are much more pronounced, as is indicated by the general yellowish grey color of the lobules on section.

## PLATES 41 AND 42.

FIG. 17. The structure of the testicle of a normal rabbit under a low power (magnified 13 diameters).

FIG. 18. Diffuse interstitial infiltration of the vaccinated testis after three days. The compression of the tubules through intertubular edema and infiltration is well shown.

FIG. 19. A four day specimen which presents a large, irregularly defined infiltration and the destruction of the tubules.

FIGS. 20 to 24. Gradual absorption of the inflammatory products with distinct diminution of tubules. There are still many foci of infiltration.

FIGS. 25 and 26. The structure of testicles which had been vaccinated three and seven weeks previously.

FIG. 27. A longitudinal section of a testicle which shrank as the result of vaccination fifty-six days previously. There are only a few tubules left, all without any spermatogenetic activities. Magnification:  $\times 13$  throughout.

#### PLATE 43.

FIG. 28. A section of normal rabbit's testicle stained by the Borrel-Calkin method (25).

FIGS. 29 and 29 a. The peculiar round or oval bodies of varying size within the affected cellular elements in the vaccinated testicles of rabbits. These bodies appear brilliantly red when stained by the Borrel-Calkin method, and they resemble the vaccine bodies so called. These bodies may have been derived from the nuclei of the cells under the influence of the vaccine virus. Magnification:  $\times 340$  throughout.

#### PLATE 44.

FIGS. 30 and 31. The structure of a normal testicle of the rabbit.

FIG. 32. An early polynuclear infiltration of the interstitial spaces of a vaccinated testicle of the rabbit after twenty-four hours.

FIG. 33. A similar but more advanced stage after three days.

FIG. 34. Extensive involvement of the testicular tubules as well as the interstitial tissues after four days.

FIG. 35. A focus where polynuclears are seen to have begun to break up after five days.

FIG. 36. An area of extensive necrosis occurring in a specimen removed after four days.

FIG. 37. The cellular infiltration and fibrin in a specimen after five days (Weigert stain).

FIG. 38. An intratubular infiltration occurring in a specimen seven days after inoculation. The cells are basophilic leucocytes. Magnification:  $\times 170$  throughout.

#### PLATE 45.

FIG. 39. A tubule completely filled with leucocytes in a specimen eight days after inoculation.

FIGS. 40 and 41. Enormous cellular infiltration along the tunica albuginea in nine day specimens.

FIG. 42. The detachment of the degenerated testicular cells from the wall of affected tubules in a twelve day specimen.

FIG. 43. A focus of infiltration in a twenty-one day specimen. Such a focus is rather rare in old specimens.



FIG. 44. A focus of infiltration which was found in a fifty-six day specimen.

FIG. 45. The general changes of the structure of the vaccinated testicle after fifty-six days.

FIG. 46. The infiltration of the tunica vaginalis in a rabbit with polynuclear leucocytes in a four day specimen.

FIGS. 47 and 48. The vaccinal reactions on the skin of rabbits. Magnification:  $\times 170$  throughout.

PLATE 46.

FIG. 49. The Guarnieri, or vaccine bodies, so called, in the corneal epithelium of a rabbit inoculated with pure testicular vaccine virus. Three day specimen.

FIG. 50. The vaccinal reaction in the skin of a rabbit inoculated with the testicular strain of vaccine virus. Stained with Calkin's modification of Borrel's method.

FIG. 51. The vaccine bodies in a rabbit's cornea inoculated with regular vaccine strain.

FIG. 52. Normal rabbit skin stained with the Borrel-Calkin method.

PLATE 47.

FIG. 53. The inoculated (left) and normal (right) testicles of bull 2 on the sixth day after the injection of pure testicular vaccine from a rabbit. The left side is seen to be somewhat larger than the right. The skin shows typical vaccinal pustules also produced with the same material.

FIG. 54. The inoculated testicle and the tunica vaginalis of the same animal, the latter showing much hemorrhage.

FIG. 55. The same organ on section. One notices the hemorrhagic foci and necrotic area.

FIGS. 56 and 57. The testicles of bull 6. The larger one was inoculated with the testicular, and the smaller with a skin strain of vaccine virus, and both were removed on the sixth day.

PLATE 48.

FIG. 58. The structure of a normal testicle of a young bull. One recognizes at once that the spermatogenesis is in full display.

FIGS. 59 to 61. The reactions following the injection of pure testicular vaccine strain into the organ. They represent changes found on the sixth day.

FIGS. 62 to 66. The vaccinal processes in the testicles of bull calves. The changes are similar to those found with young bulls and rabbits, except that the animals were too young to have any spermatogenesis, even in the unaffected areas of the organ. Both in the young bulls and in the bull calves the interstitial infiltration with polynuclear leucocytes is marked. Magnification:  $\times 170$  throughout.

PLATE 49.

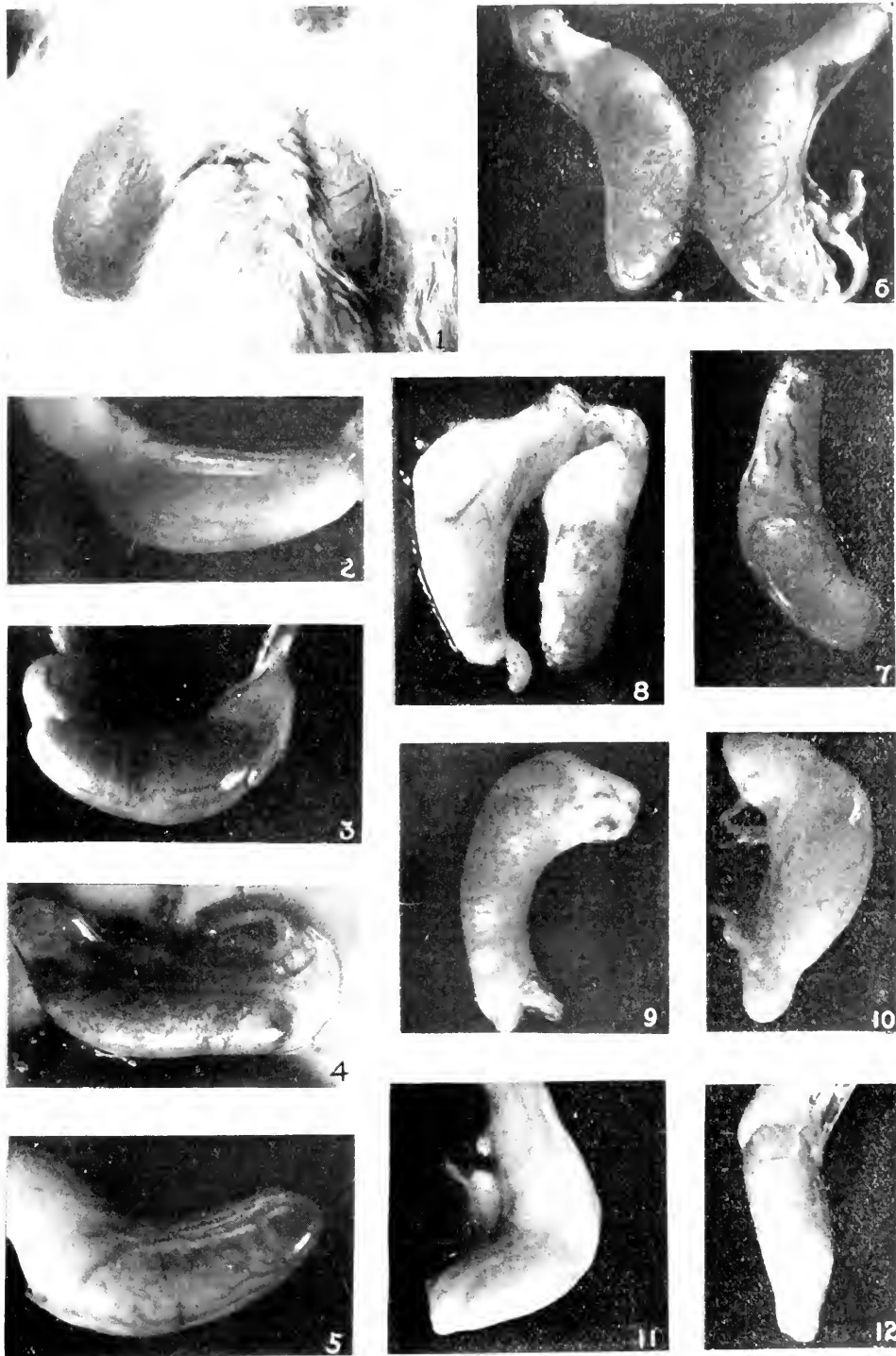
FIG. 67. The typical vaccinal effect on the skin of a rabbit inoculated with a pure testicular strain of vaccine virus on the sixth day.

FIG. 68. A confluent eruption on the skin of a rabbit vaccinated with a pure testicular strain.

FIG. 69. The typical vaccinal effect on the skin of a calf inoculated with a pure testicular strain of vaccine virus obtained from a rabbit, on the sixth day. Natural size.

PLATE 50.

FIGS. 70 and 71. The fifth and the sixth day phases of the first vaccination in a child three years and two months old. The virus was prepared in the rabbit according to the method described in this work, and was absolutely free from bacteria. Notice the uninterrupted linear eruption on both sides. In figure 71 the mottled appearance around the site of vaccination is due to the adhesion of the vaccine shield.



(Noguchi) Cultivation of Vaccinia Virus

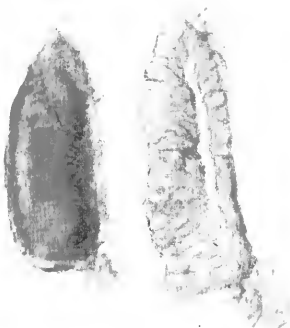




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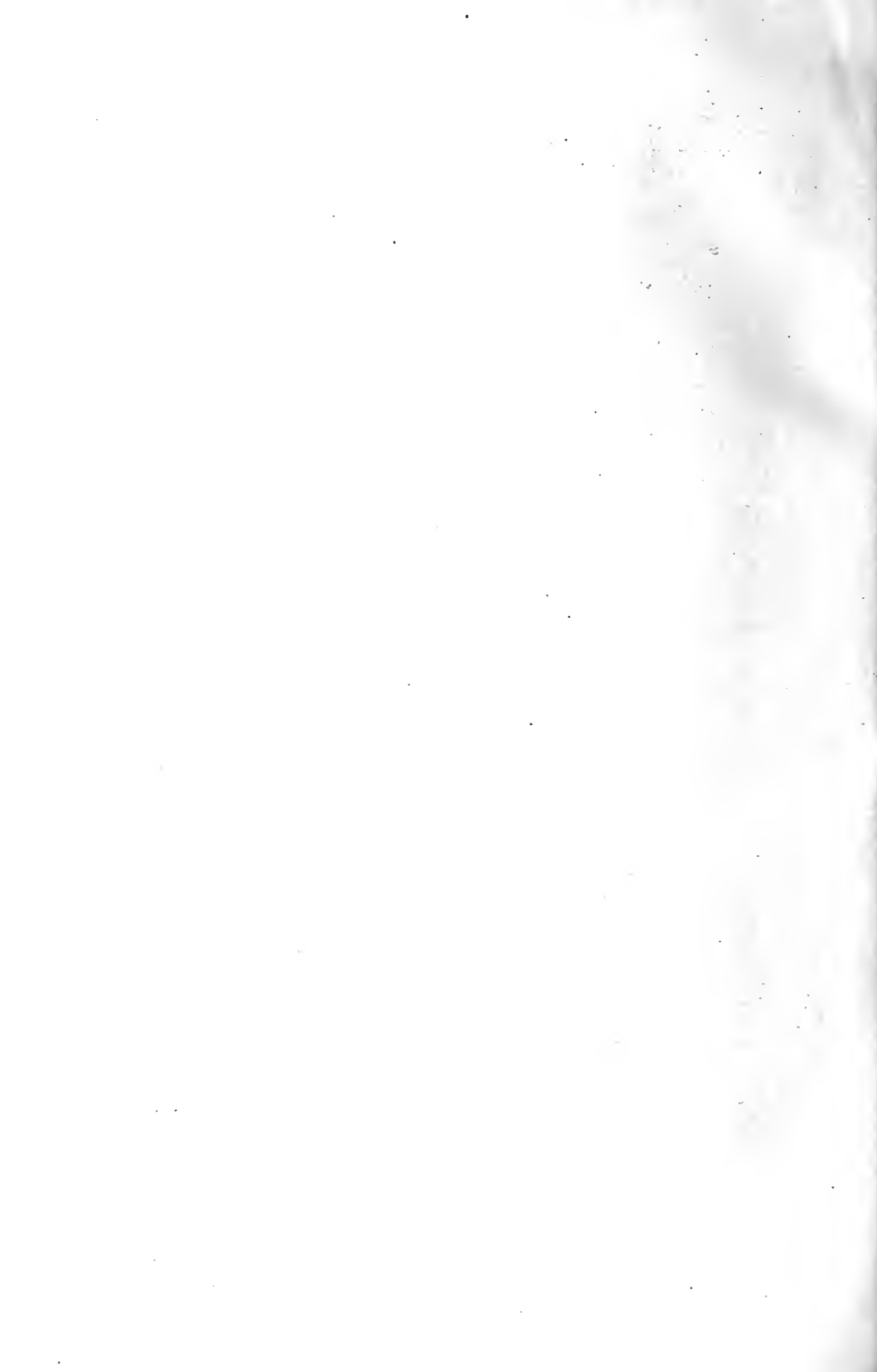
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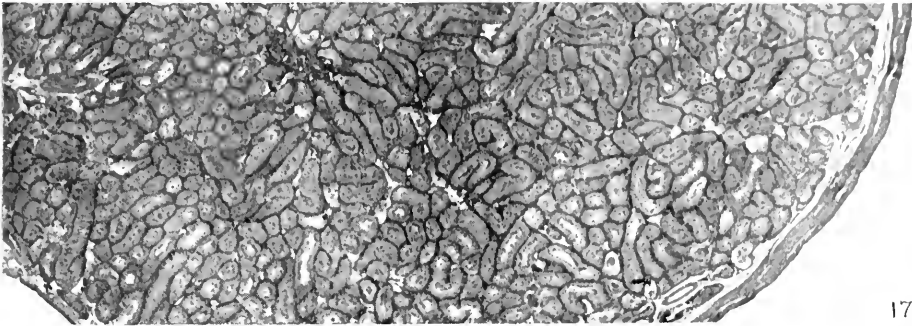


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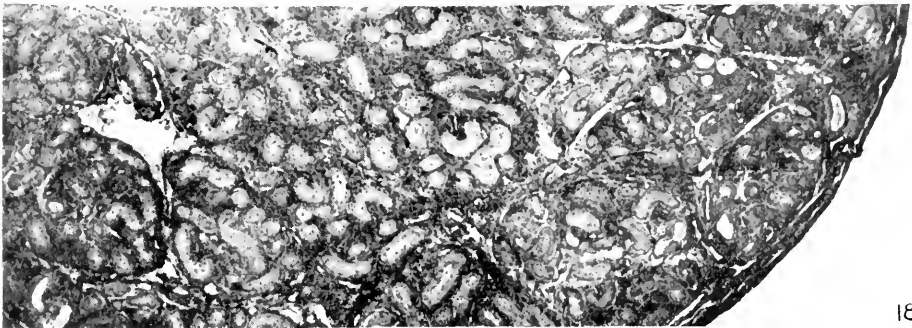


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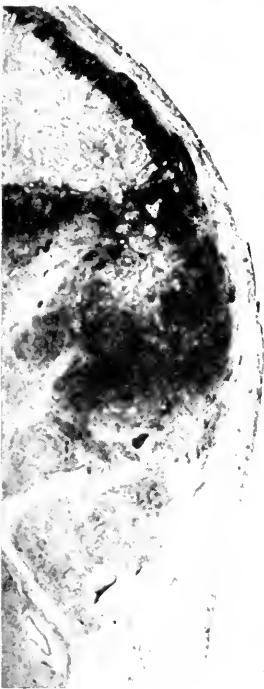




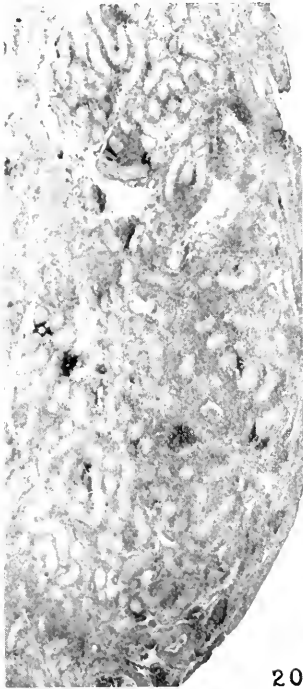
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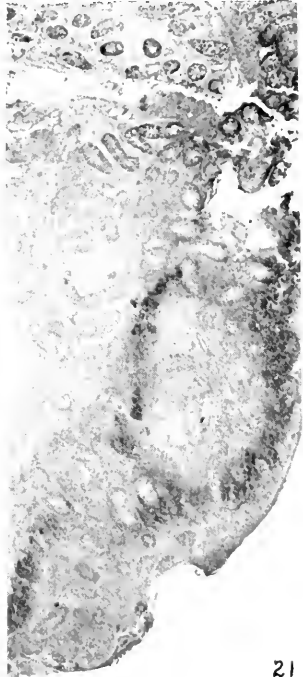
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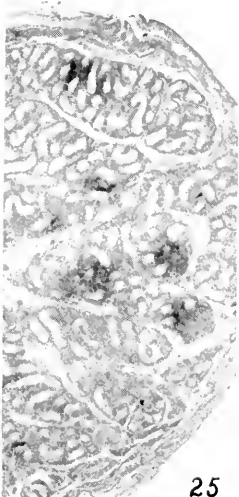
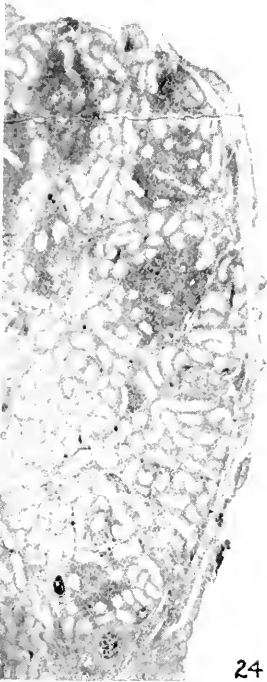
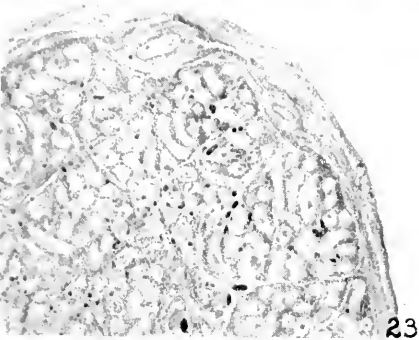
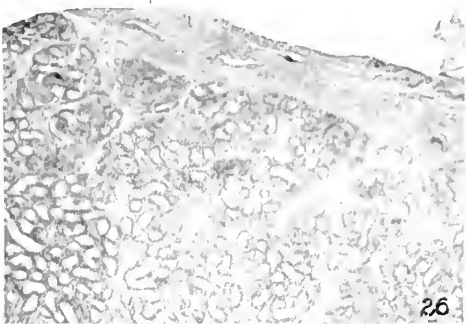
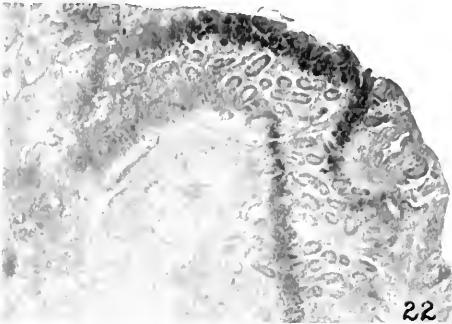
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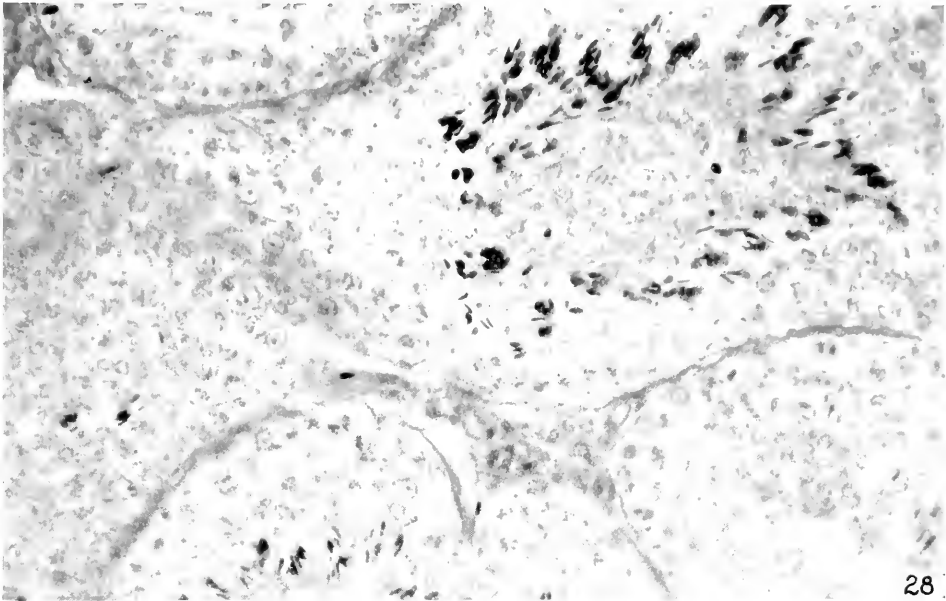
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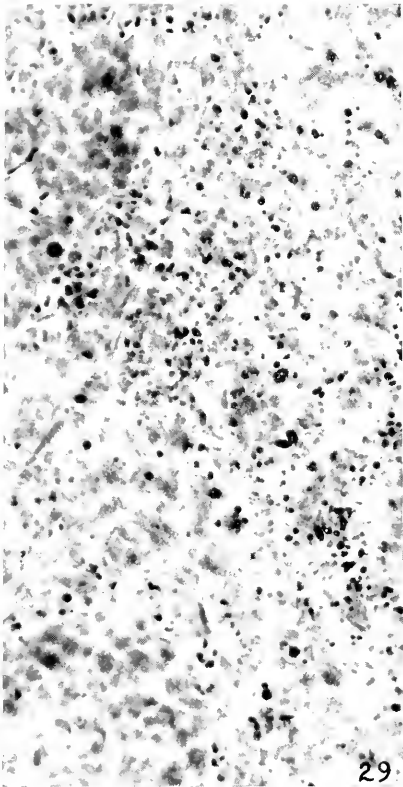




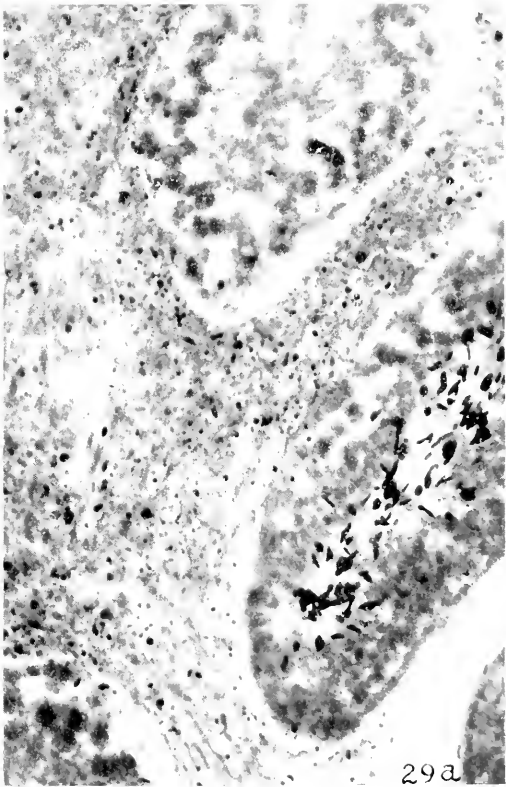




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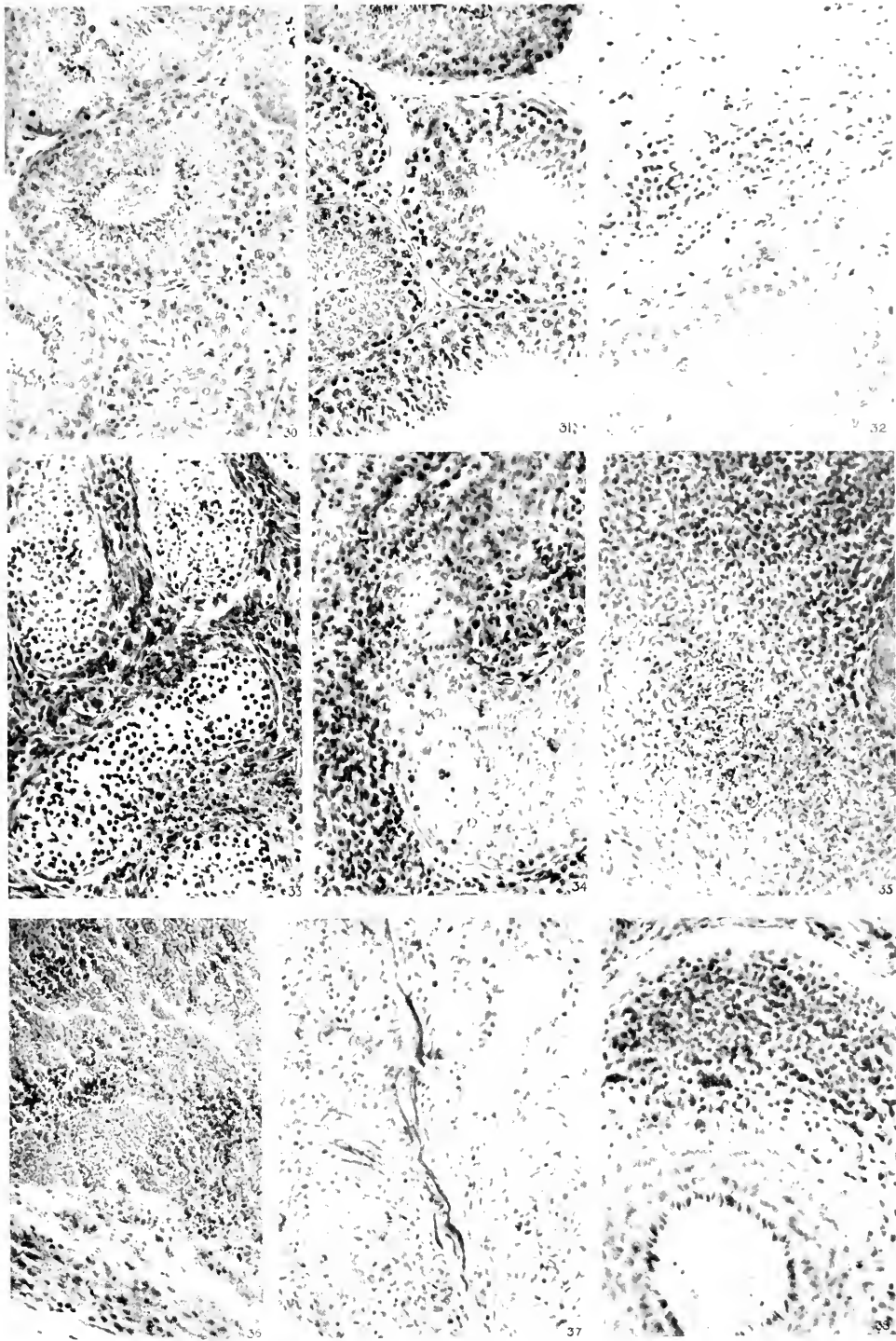


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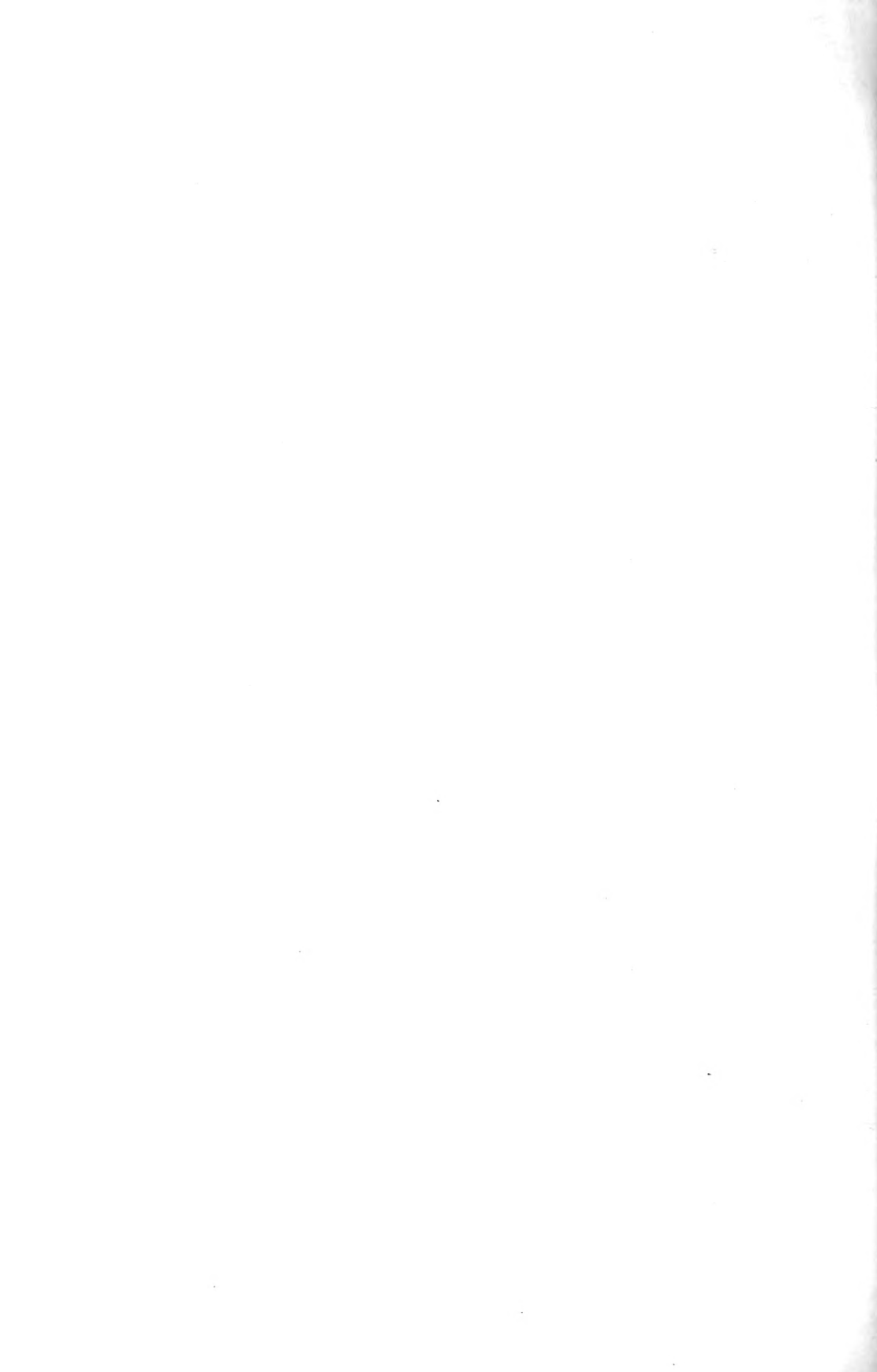


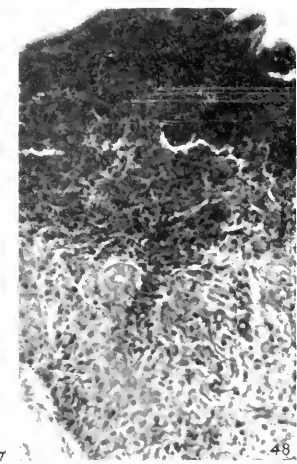
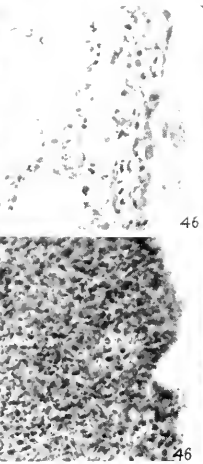
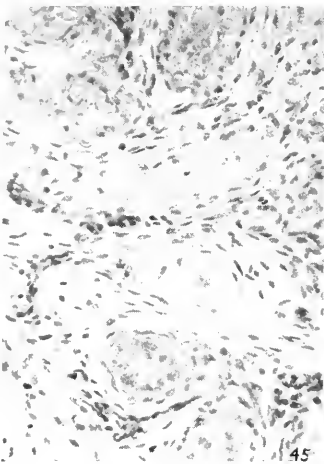
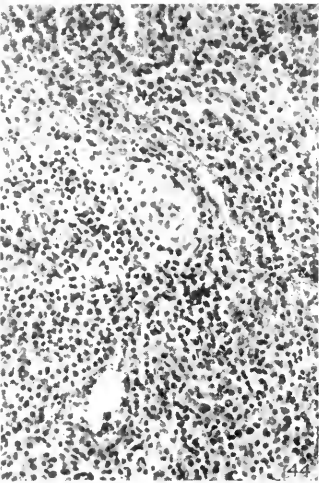
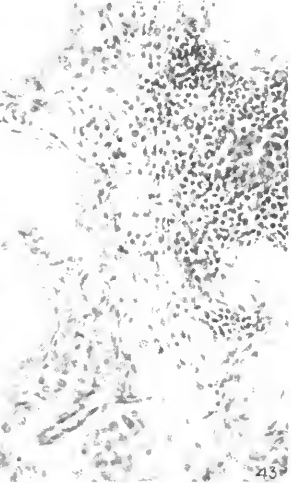
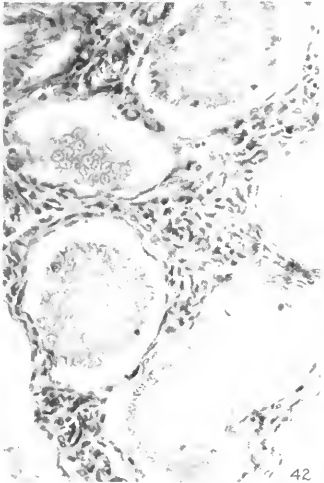
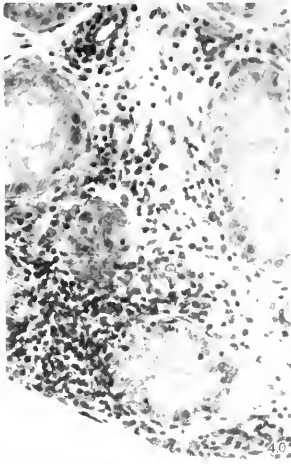
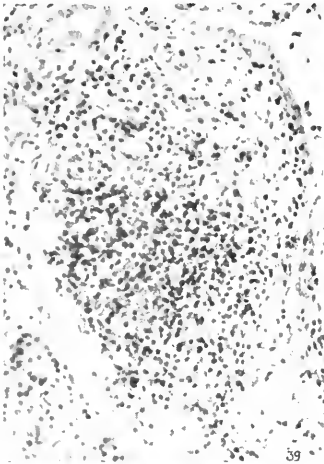
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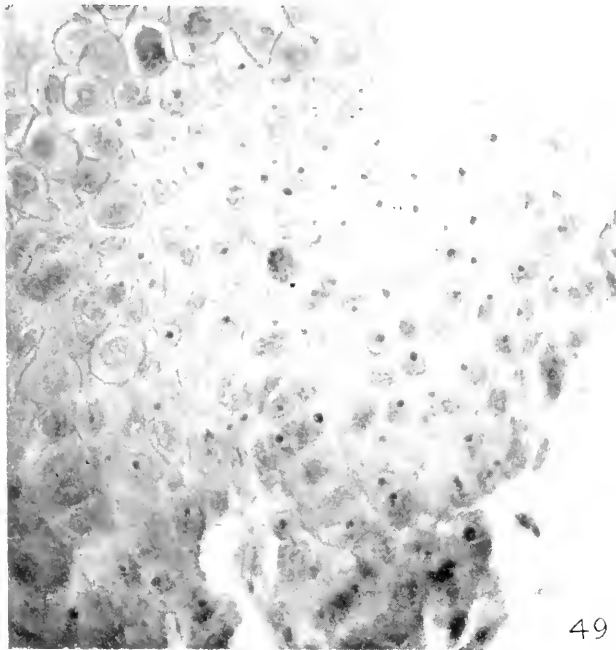
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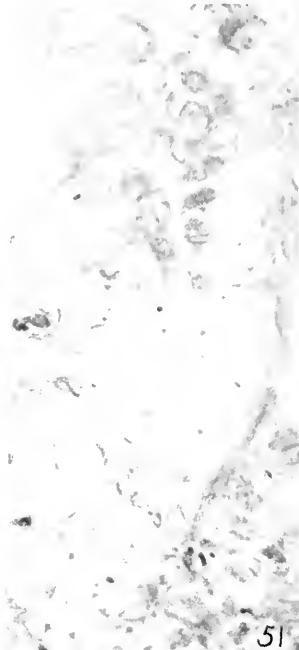








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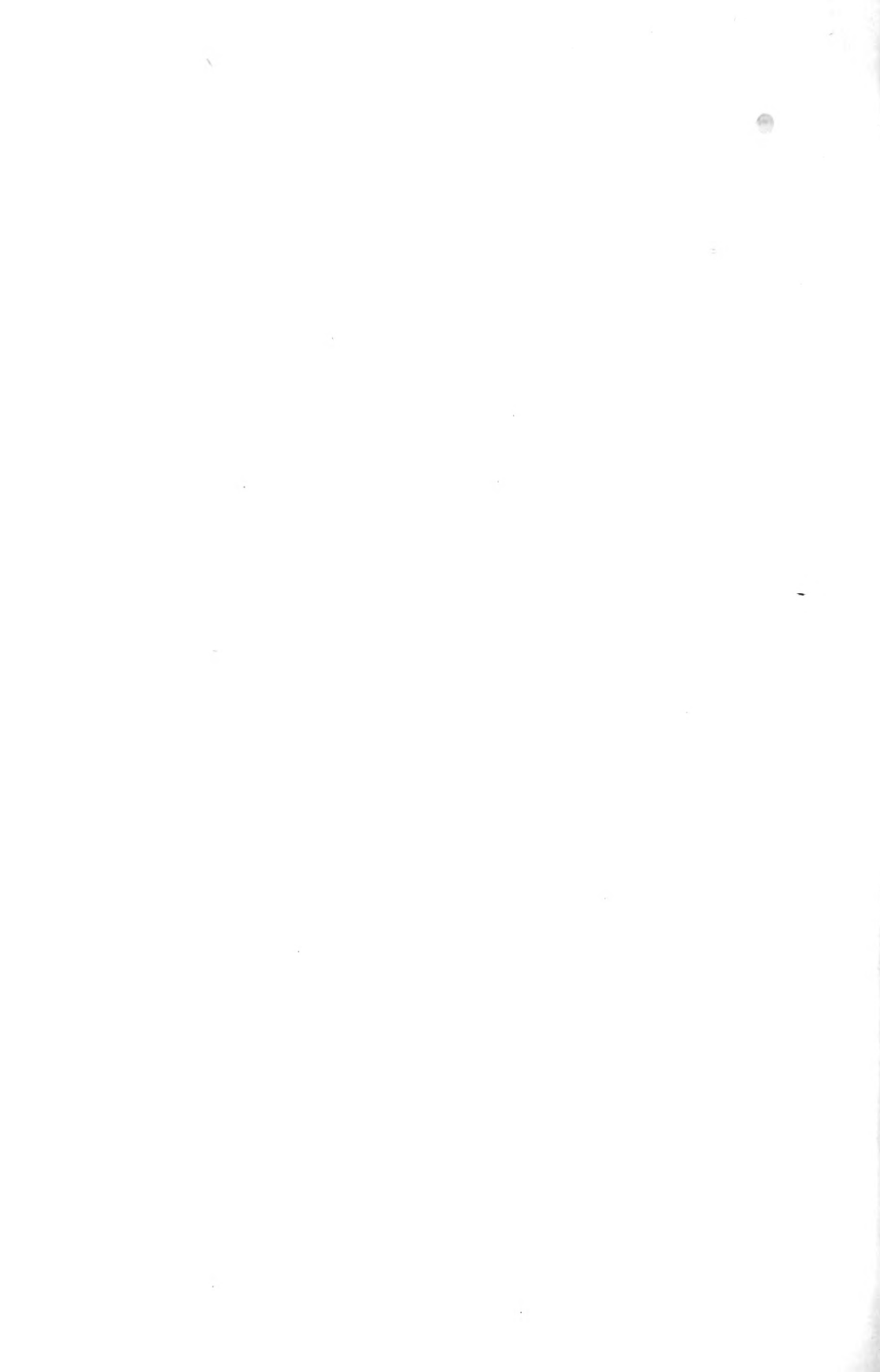
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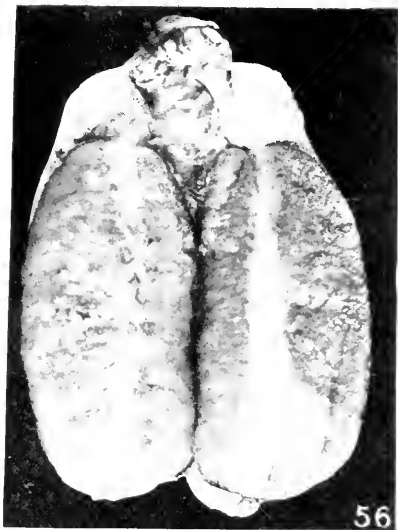


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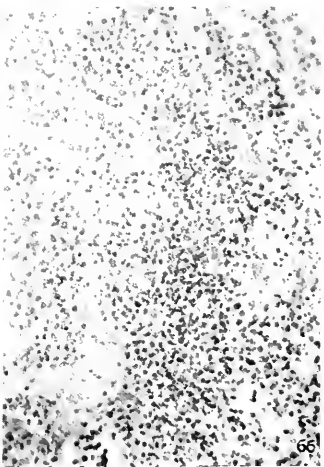
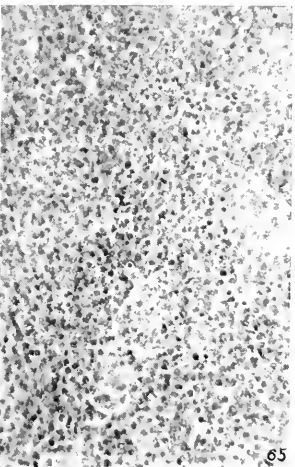
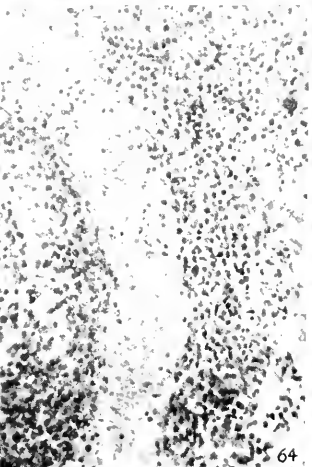
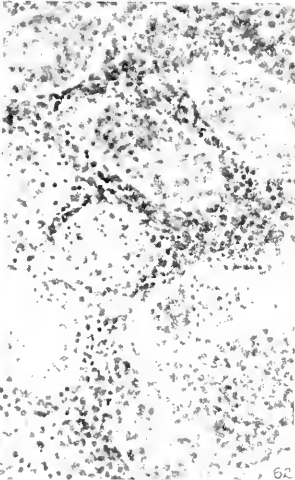
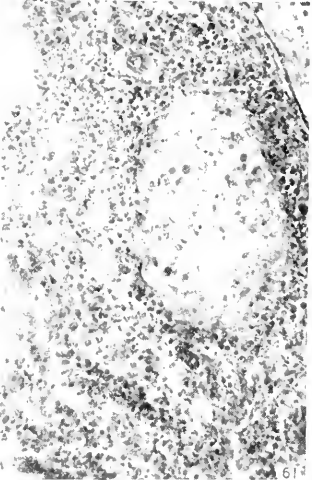
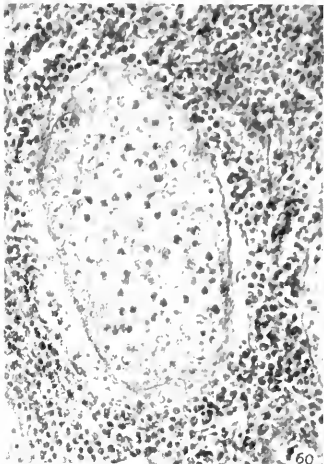
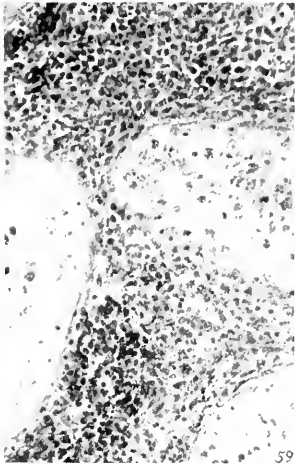
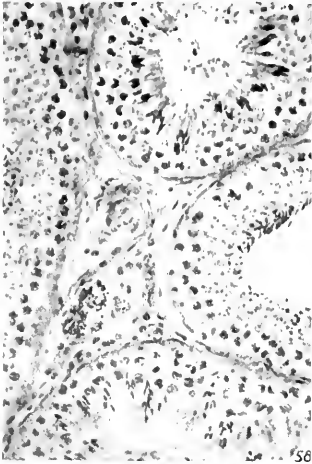


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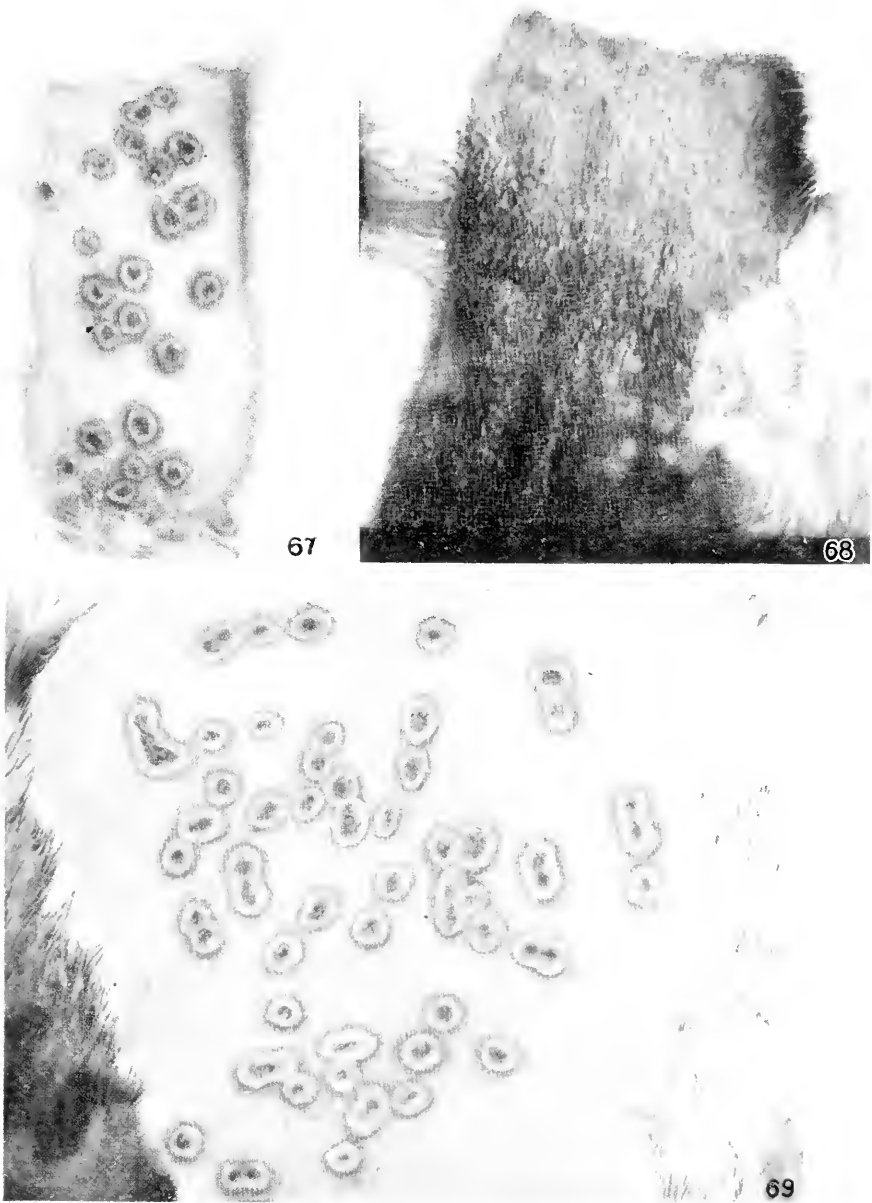




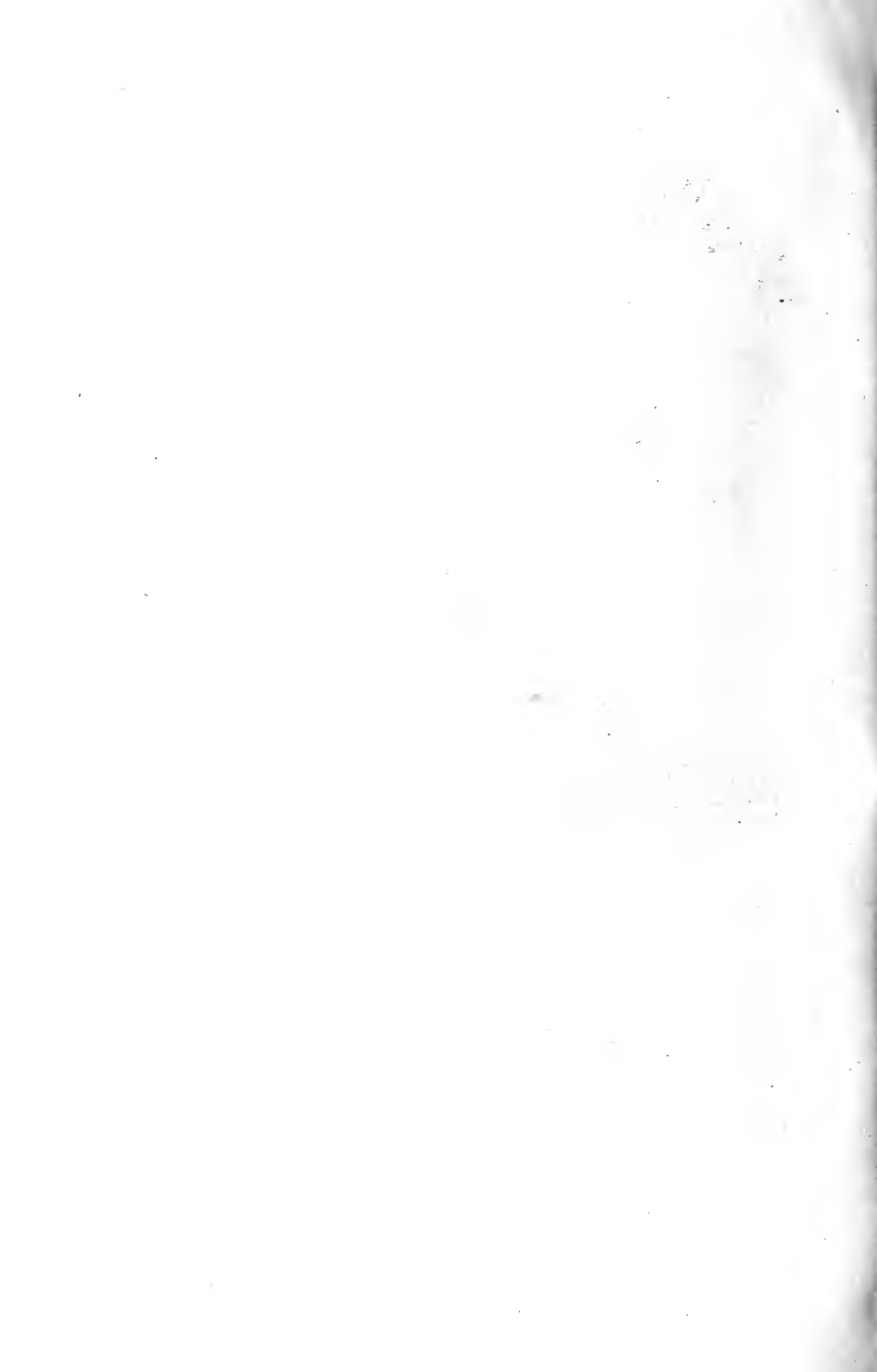


(Noguchi: Cultivation of Vaccine Virus.)

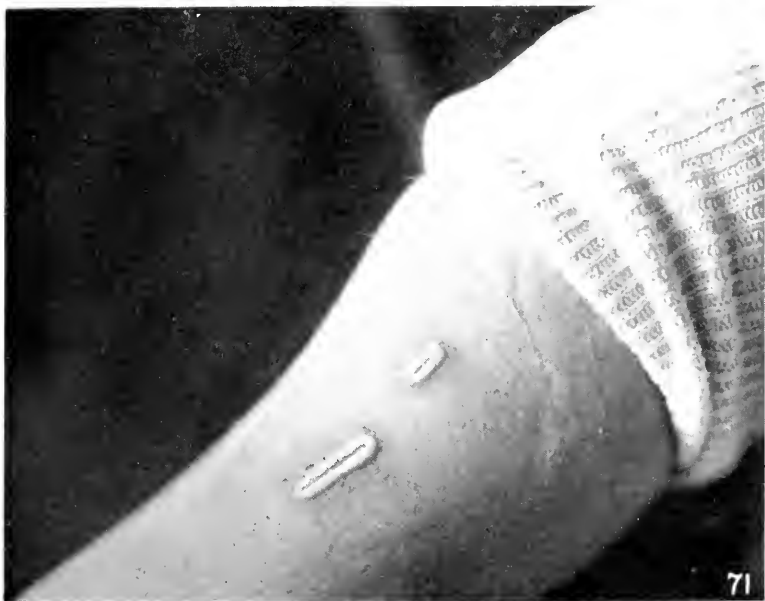




(Noguchi: Cultivation of Vaccine Virus.)







(Noguchi: Cultivation of Vaccine Virus.)



## THE TRANSPLANTATION OF TUMORS TO FOREIGN SPECIES.\*

By CASIMIR FUNK, D.Sc.

PLATE 51.

It is known that when a mouse tumor is implanted into rats growth goes on for seven to ten days, but then resorption takes place. The failure of tumors to grow in animals of foreign species seems to be a general rule to which few exceptions are known.<sup>1</sup> Ehrlich explains the temporary growth of mouse tumors in rats on the theory, the so called athreptic theory, that the foreign organism is lacking in specific foods, which exist only in animals of the sort from which the tumor came.

The recent work on the influence of diet on tumor growth<sup>2</sup> has shown the importance of this hitherto undervalued factor. It seems advisable to investigate the influence of feeding to animals in which a tumor of a foreign species is growing, the tissue of that species, more especially tumor tissue itself. In this way the substance which the tumor needs might be supplied. Some time ago I tried to render rats more susceptible to mouse tumor by injections of alcoholic extracts of mouse tumor, but though some success was obtained the study had to be abandoned as the injections proved toxic.

The experiments here to be reported were arranged as follows: The tumor chosen was Ehrlich's mouse chondroma, which "takes" in a very high percentage of the mice to which it is transplanted. Before the experiments were started, many mice were inoculated with the growth to supply the tumor material used for feeding. Then a

\* Received for publication, February 15, 1915.

<sup>1</sup> Among the reported cases of successful transplantation of human tumors to animals, or of animal tumors to other species, few will stand criticism. In very few cases indeed (Dagonet-Mauclair, Carl Lewin) were the growths successfully reinoculated.

<sup>2</sup> Sweet, J. E., Corson-White, E. P., and Saxon, G. J., *Jour. Biol. Chem.*, 1913, xv, 181. Funk, C., *Lancet*, 1914, i, 98. Rous, P., *Jour. Exper. Med.*, 1914, xx, 433.

large number of rats were inoculated,—also with the chondroma,—and these were divided into two batches, one batch being fed on ordinary food, the other on ordinary food with the twice daily addition of freshly excised chondroma from the mice. The rats ate the tumor material with avidity.

The results were striking. In rats fed the tissue of the chondroma, this growth succeeded on inoculation much better than in those receiving ordinary diet.

*Experiment I. First Generation.*—Two batches, each of thirty young rats, were employed. One batch was fed, besides ordinary food, the mouse chondroma twice each day. After a week of the feeding both batches were inoculated with material of the mouse chondroma. The same tumor was used for all the inoculations. In the controls fed on ordinary food but one small tumor appeared. This was noticed after ten days and was completely absorbed after fifteen days. Of the rats fed the chondroma, six developed tumors, and in one instance the growth after sixteen days was about 12 by 18 mm. in size. It was disc-shaped and only 3 to 4 mm. thick. This tumor was examined microscopically and showed the histological characters of the original tumor (figure 1), but in distinction from the latter, which was soft and hemorrhagic, it was firm, and consisted, as the drawing shows, practically entirely of the chondroma capsules with very little homogeneous, intracellular substance (figure 2). Bits of it were transplanted to twenty rats and six mice. A chondroma developed in all of the latter, but much later than usual, showing that the transplantation into rats had in some way rendered the tumor unfit for mice. The results of the transplantation into rats is recorded below.

In another rat of this experiment the tumor reached, after five weeks, the size of a pea and was reinoculated into six mice with success in all cases.

*Second Generation.*—Twenty rats were inoculated with bits of the large tumor just described. All were fed the mouse chondroma. In one of the eighteen which survived seventeen days there was noted a tumor measuring 1 by 0.5 cm. Two other rats showed after twenty-four days tumors of the size of a small pea; and six had very small tumors which finally disappeared. The largest growth was examined microscopically and proved to be the chondroma. It was transplanted to eight rats and four mice,—to all of the latter successfully. The result in the rats was as follows:

*Third Generation.*—The eight rats inoculated with the tumor of the second generation were all fed the mouse chondroma. After eleven days one had a tumor the size of a big pea and five had smaller nodules, which finally disappeared. The largest tumor in this case was found microscopically to be a chondroma.

Further transplantations were abandoned because there was not sufficient material for inoculation.

*Experiment II.*—Twenty rats were inoculated with the mouse chondroma from a mouse of experiment I, which had been inoculated with a tumor from

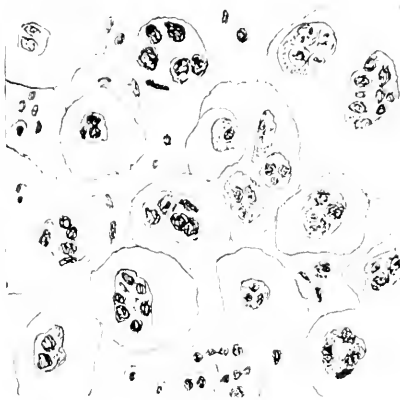


FIG. 1.

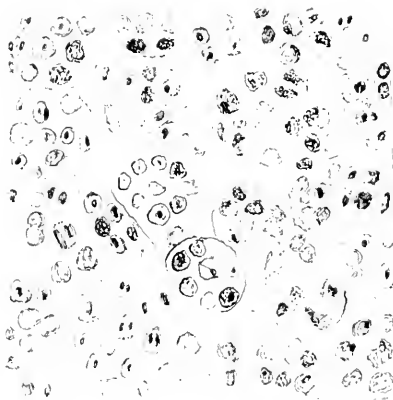


FIG. 2.

(Funk: Transplantation of Tumors to Foreign Species.)



a rat of the first generation. The animals were divided into two batches, one fed on ordinary food, the other on the same diet with the addition of tumor tissue. In the latter batch two tumors developed which still persisted after twenty-four days. One was the size of a large pea. In the controls no tumors were observed.

The experiments show that by feeding tumor tissue to animals of alien species they may be rendered more favorable to the growth of this tumor. The tumor employed, the mouse chondroma, was successfully transplanted in three successive series of rats, and kept alive for five weeks in all. But with every transplantation the tumors obtained were smaller, until the material was not sufficient for inoculation purposes. The results might have been more successful if a larger number of animals had been used, since only a very small percentage of the rats furnished tumors sufficiently large to be useful for transplantation. The method is promising enough for further experiments on other perhaps more suitable tumors and on other animals.<sup>3</sup>

#### SUMMARY.

By feeding to rats the tissue of the mouse chondroma they are rendered more suitable as hosts for the growth. Using this method rats have been inoculated successfully with the mouse tumor and it has been transferred to three successive series of these animals.

#### EXPLANATION OF PLATE 51.

FIG. 1. Mouse tumor (chondroma). Leitz ocular 2, objective 8.

FIG. 2. Mouse tumor (chondroma) in a rat of experiment I, generation I. Leitz ocular 2, objective 8.

<sup>3</sup> Following the work of Murphy (Murphy, Jas. B., *Jour. Exper. Med.*, 1913, xvii, 482) on heterogeneous transplantation in embryos, Bashford has shown that newly born animals are favorable hosts for the tumors of other species (Bashford, E. F., *Twelfth Annual Report of the Imperial Cancer Research Fund*, 1913-14, 9). The animals used in the present experiments were one-half to three-quarters grown.

## THE EFFECT OF ARSENIC COMPOUNDS ON THE ROUS CHICKEN SARCOMA.\*

By CASIMIR FUNK, D.Sc.

The chemotherapy of cancer, a subject much studied of late, has been investigated up to the present with rat and mouse tumors only. The results thus far obtained are contradictory, perhaps for the reason that in rats and mice a spontaneous disappearance of tumors is not infrequent, while, furthermore, diet and environment markedly influence the rate of tumor growth.

The author decided to perform some chemotherapeutic experiments with the Rous spindle celled chicken sarcoma, because, in his hands at least, this growth has never undergone spontaneous retrogression. Various arsenic compounds were selected for the test, since arsenic has shown itself to be useful in the treatment of cutaneous sarcomas. Moreover, arsenic is of use in certain infections; and there is evidence for the view that the chicken sarcoma is of an infectious nature.<sup>1</sup> For such reasons a curative action of arsenic seemed at least possible.

For the experiments chickens four to six weeks old of the Plymouth Rock variety were used, and the tumor was implanted in the pectoral muscle of one side. The arsenic compounds employed were arsenic and arsenious acids, cacodylic acid, atoxyl, and neosalvarsan. Fowls, as Ehrlich<sup>2</sup> has shown, withstand much higher doses of arsenic than do mammals. Large doses were administered intramuscularly every second day: for the first experiment into the pectoral muscle containing the tumor, but not into the tumor itself; for the second experiment into the thigh. A slightly beneficial effect was observed as a result of the intrapectoral injections, but the injections into the thigh had no evident result on the tumor.

\* Received for publication, February 15, 1915.

<sup>1</sup> Rous, P., and Murphy, Jas. B., *Jour. Am. Med. Assn.*, 1912, lviii, 1938.

<sup>2</sup> Ehrlich, P., and Hata, S., *Die experimentelle Chemotherapie der Spirillosen*, Berlin, 1910.



## EXPERIMENTAL.

*Experiment I.*—Six batches of thirteen chickens each were inoculated with the Rous sarcoma. Three days after the inoculation the treatment was begun and the injections were repeated every second day, the doses being as follows:

Arsenic acid .....	0.1 mg.
Arsenious acid .....	0.1 mg.
Cacodylic acid .....	20 mg.
Atoxyl .....	10 mg.
Neosalvarsan .....	50 mg.

The injections were made into the pectoral muscle containing the tumor. The results of this experiment were as follows:

Of the control fowls none lived 18 days after inoculation.  
 Of those treated with arsenic acid 2 lived 18 days after inoculation.  
 Of those treated with arsenious acid 6 lived 18 days after inoculation.  
 Of those treated with cacodylic acid 2 lived 18 days after inoculation.  
 Of those treated with atoxyl 3 lived 18 days after inoculation.  
 Of those treated with neosalvarsan 2 lived 18 days after inoculation.

In this experiment the injection of arsenic compounds seemed to have a slight inhibiting effect on the tumor growth, especially in the case of arsenious acid, but in no case was a cure effected.

*Experiment II.*—In this experiment the tumor was inoculated as usual into the pectoral muscle and the arsenic compounds were injected into the muscle of the leg. The treatment was begun two days before the inoculation of the tumors and was repeated every second day.

Controls.	No. inoculated.	Survived 20 days.	Survived 27 days.
Controls	27	6	2
Arsenic acid	29	7	4
Arsenious acid	29	7	0
Cacodylic acid	29	9	2
Atoxyl	29	7	0
Neosalvarsan	30	5	1

The rapidity of growth of the tumors was the same in the treated fowls as in the controls, but in the former the tumor ulcerated more quickly.

## SUMMARY.

Arsenic and arsenious acids, cacodylic acid, atoxyl, and neosalvarsan fail to influence markedly the growth of the Rous chicken sarcoma.

## ANTIBODY FORMATION AGAINST TREPONEMA PALLIDUM—AGGLUTINATION.\*

BY HANS ZINSSER, M.D., AND JOSEPH GARDNER HOPKINS, M.D.

(From the Department of Bacteriology of the College of Physicians and Surgeons, Columbia University, New York.)

PLATES 52 TO 54.

Although active immunization in syphilis has been extensively attempted by many workers, the results seem to show generally unsuccessful experiments. It is the opinion of Neisser and others that true immunity in syphilis has not so far been demonstrated and possibly does not exist. It seems that a human being is immune to reinoculation only while still diseased, but that susceptibility is again established after clinical recovery. As regards animals experimentally inoculated these matters are still uncertain, because of the unavoidable technical difficulties; for in none of the animals easily and cheaply accessible to most laboratory workers can syphilis be produced in anything like the generalized or prolonged course observed in human beings.

It has been the aim of many workers to attempt the demonstration of antibodies in the circulation of animals experimentally inoculated or treated with extracts of either syphilitic tissues or treponema culture material. Before the cultivation of *Treponema pallidum* by Noguchi's methods had made possible extensive experimentation with pure cultures, many investigators attempted active immunization of animals with tissues rich in treponemata, but with entirely unsatisfactory results.

Neisser<sup>1</sup> and Bruck summarize their opinion as follows: "It is plain, therefore, that parasitocidal antibodies do not occur in the course of syphilis, and that we may at most count only upon the occurrence of specific complement-fixing and agglutinating substances, although the occurrence of such antibodies can be determined with accuracy only when we shall be able to work with pure cultures of treponemata."

\* Received for publication, February 20, 1915.

<sup>1</sup> Neisser, A., *Arb. a. d. k. Gsndhtsamte.*, 1911, xxxvii, 187, 206.

In a later summary Bruck<sup>2</sup> states that complement fixation in syphilis can be looked upon as only in part depending upon a specific antibody reaction. He states also that Fornet and Schereschewski's demonstration of precipitins in syphilitic sera cannot be confirmed, and that the formation of true agglutinins could not be determined by Uhlenhuth and Mulzer, who treated rabbits, goats, and monkeys with materials containing considerable amounts of treponemata. He adds, however, that this question will probably remain unsettled until the work can be repeated with materials richer in microorganisms.

Noguchi,<sup>3</sup> in April, 1912, published a paper on the fixation of complement when aqueous extracts of syphilis cultures were used as antigen, and the sera of patients with syphilis and of experimentally inoculated rabbits were used. He found positive fixation in the majority of the cases of secondary, tertiary, and hereditary syphilis, also in one of five syphilitic rabbits, using as antigen the emulsion from syphilitic testicles or from pure cultures. The validity of the reactions obtained with syphilitic testicles he regarded as doubtful, since emulsions from normal testicles gave, with some of the sera, equally strong reactions.

Craig and Nichols,<sup>4</sup> using alcoholic extracts of pure cultures of the syphilis organism and some other spirochete, found occasional fixations in syphilitic cases, but these were weaker than similar fixations obtained with the ordinary lipid tissue extracts used in the common Wassermann technique.

Kolmer, Williams, and Laubaugh,<sup>5</sup> working both with aqueous and alcoholic extracts of concentrated pure cultures, found that such antigens gave positive fixation reactions with the serum of a considerable number of human syphilitic cases, and strong reactions with the serum of rabbits treated with treponema cultures.

Kolmer<sup>6</sup> also observed that treponemata from cultures in horse serum broth were agglutinated by the sera of rabbits which had received injections of such cultures either intravenously or into the testicle.

These later researches seem to indicate definitely that circulating antibodies may be formed in the course both of spontaneous syphilis of human beings and in animals treated with cultures of the treponema. Noguchi in summing up the problem in his paper concludes that his experiments seem to show that the ordinary Wassermann reaction is not determined by specific antibodies; that, however, the fixation produced with the culture pallida antigen seems to signify the presence of the true antibodies. He remarks upon the peculiar fact that the concentration of antibodies in syphilitic patients as determined by the pallida antigen seems to be surprisingly slight when compared with antibody formation in many other infectious diseases.

<sup>2</sup> Bruck, C., in Kolle, W., and von Wassermann, A., *Handbuch der pathogenen Mikroorganismen*, 2d edition, Jena, 1913, vii, 1060.

<sup>3</sup> Noguchi, H., *Jour. Am. Med. Assn.*, 1912, lviii, 1163.

<sup>4</sup> Craig, C. F., and Nichols, H. J., *Jour. Exper. Med.*, 1912, xvi, 336.

<sup>5</sup> Kolmer, J. A., Williams, W. W., and Laubaugh, E. E., *Jour. Med. Research*, 1913, xxviii, 345.

<sup>6</sup> Kolmer, J. A., *Jour. Exper. Med.*, 1913, xviii, 18.

That specific circulating antibodies for *Treponema pallidum* may be formed in syphilitic human beings and in experimentally inoculated animals has thus been rendered likely and has to some extent found support in experimental observation.

The observations which we desire to report in the present communication deal entirely with the appearance of specific agglutinating antibodies in the sera of rabbits intravenously treated with emulsions in salt solution of *Treponema pallidum*, killed by heating to 56° C. for thirty minutes. This temperature was chosen after it had been ascertained by Dr. Hopkins (in experiments as yet unpublished) that the thermal death point of these microorganisms lay between 50° and 55° C.

We have been studying antibody formation in syphilitic infection for some time, but our work could not be carried out on a sound experimental basis until we had developed a technique by which we could obtain larger quantities of treponema material which could be centrifugalized and washed free of culture fluid in the same way as this is done in similar experiments with bacteria. These methods have been in essence described in a previous paper.<sup>7</sup>

In all our antibody work in which the serum of treated rabbits was used, we took care to treat animals with treponema material that had been grown in sheep serum mixtures with salt solution or broth, whereas the treponemata used in the final reactions were grown on human ascitic fluid media. Such a technique was used in order to avoid the possibility of false reactions which might have been caused by the production of protein antibodies by antigen injected into the animals with remnants of the culture fluids. This is a precaution which we think should be taken in all experiments with this micro-organism.

The material for agglutination was obtained by decanting the culture fluid from 250 cubic centimeter flasks in which the treponemata had been grown for from four to six weeks. Relatively slow centrifugation was first carried out for a short time to throw down coarse particles. After this, rapid and prolonged centrifugation yielded a sediment which when suspended in salt solution gave an evenly turbid emulsion very rich in treponemata. It should be noted

<sup>7</sup> Zinsser, H., Hopkins, J. G., and Gilbert, R., *Jour. Exper. Med.*, 1915, xxi, 213.

for the benefit of others undertaking these experiments that it is often difficult to free the treponema emulsion entirely from minute particles of precipitate likely to occur in old ascitic fluid mixtures. Such a precipitate often adds considerable turbidity to the final emulsion and may result in disturbing irregularities of reaction. In many of our experiments it will be noticed that  $\pm$  is used, and this often signifies very slight flake formation in the tubes when such an unclean suspension was used. The reactions are as sharp and distinct as similar reactions on the agglutination of bacteria when the emulsions are unmixed with such precipitate. We have found it of advantage to discard material in which such a disturbing precipitate was present to any extent. When, as in a very few experiments, spontaneous agglutination occurred in salt solution this was of extremely slight degree only and of such a character that it was easily and sharply differentiable from that occurring in the positive tubes.

## EXPERIMENT I.

*Suspension Strain A (Macroscopic).*

*Serum of Rabbit 609 (Intravenously Treated with Five Injections of Suspension A Ranging from 1 to 5 Cubic Centimeters).*

*Each Tube Contained 0.5 of a Cubic Centimeter of Serum Dilution and 0.25 of a Cubic Centimeter of Suspension.*

Concentration.	Serum 609. First bleeding.		Serum 609. Second bleeding.		Normal rabbit serum.	
	2 hrs.	15 hrs.	2 hrs.	15 hrs.	2 hrs.	15 hrs.
Undiluted	+++	+++	+++	+++	+++	+++
1 : 2	+++	+++	+++	+++	+++	+++
1 : 5	+++	+++	+++	+++	+++	+++
1 : 10	+++	+++	+++	+++	+++	+++
1 : 20	+++	+++	+++	+++	$\pm$	$\pm$
1 : 50	+++	+++	+++	+++	0	0
1 : 100	+++	+++	+++	+++	0	0
1 : 200	+++	+++	+++	+++	0	0
1 : 500	++	+++	+++	+++	0	0
1 : 1,000	$\pm$	0	Not set up		0	0
1 : 2,000	0	0			0	0
1 : 4,000	0	0			0	0
Salt solution (control)	0	0	0	0	0	0

## EXPERIMENT II.

*Suspension Strain A (Macroscopic).*

*Serum of Rabbit 611 (Intravenously Treated with Six Injections of Suspension A Ranging from 1 to 4 Cubic Centimeters).*

*Each Tube Contained 0.5 of a Cubic Centimeter of Serum Dilution plus 0.25 of a Cubic Centimeter of Treponema Suspension.*

Concentration.	Serum 611.		Normal rabbit serum.	
	2 hrs.	15 hrs.	2 hrs.	15 hrs.
Undiluted	+++	+++	+++	+++
1 : 2	+++	+++	+++	+++
1 : 5	+++	+++	+++	+++
1 : 10	+++	+++	+++	+++
1 : 20	+++	+++	±	±
1 : 50	+++	+++	0	0
1 : 100	+++	+++	0	0
1 : 200	+++	+++	0	0
1 : 500	+++	+++	0	0
1 : 1,000	+++	+++	0	0
1 : 2,000	±	+++	0	0
1 : 4,000	0	±	0	0
Salt solution (control)	0	0	0	0

Since it seemed important to establish, if possible, the ordinary limits of the agglutinating properties of normal rabbit serum, we carried out the following experiment with a larger number of normal rabbit sera and a suspension of strain A. For comparison, rabbit sera 609 (five injections), 610 (five injections), and 619 (five injections) were set up with the same suspension.

## EXPERIMENT III.

Concentration.	Serum 609.	Serum 610.	Serum 619.	Normal 1.	Normal 2.	Normal 3.	Normal 4.	Normal 5.	Normal 6.	Normal 7.
Undiluted	+++	+++	+++	++	++	+++	++	++	+++	++
1 : 10	+++	+++	+++	±	±	+	±	±	±	±
1 : 20	+++	+++	+++	±	±	+	±	±	±	±
1 : 50	+++	+++	+++	±	±	+	±	±	+	±
1 : 100	+++	+++	+++	±	±	+	±	±	+	±
1 : 200	+++	+++	+++	±	±	+	±	±	+	±
1 : 500	+++	+	+++	±	±	+	±	±	+++	±
1 : 1,000	+	+	+++	±	±	+	±	±	+	±
1 : 2,000	+	+	+++	±	±	+	±	±	+	±
1 : 4,000	+	+	±	±	±	+	±	±	+	±
Salt solution (control)	±	±	±	±	±	+	±	±	±	±

In interpreting this experiment it is necessary to note that the ±, or very slight flocculation, which occurred throughout in the normal

sera, occurred also in the salt solution controls. It must, therefore, be looked upon as spontaneous clumping, in our opinion due, in part at least, to the fact that the emulsion of treponemata on this day contained not inconsiderable amounts of the precipitate granules mentioned above. Moreover, this flocculation in the normal sera and in the salt solution was slow and in character recognizably different from that appearing in the tubes with immune serum, in that in the last named the flakes formed rapidly and settled out in a short time.

The irregularity in normal serum 6 we cannot explain, but feel that in spite of it our results are sufficiently convincing.

Since at this time we happened to possess good material from a mass culture of one of the strains which Dr. Noguchi had kindly sent us, his No. 9, our Noguchi I, we carried out an experiment to determine whether the serum of a rabbit treated with our strain A would agglutinate Noguchi I.

#### EXPERIMENT IV.

*Suspension Strain Noguchi I. Serum of Rabbit 609.*

*Each Tube Contained 0.5 of a Cubic Centimeter of Serum Dilution and 0.25 of a Cubic Centimeter of Spirochete Suspension.*

Concentration.	Serum 609. First bleeding.		Serum 609. Second bleeding.		Normal rabbit serum.	
	2 hrs.	15 hrs.	2 hrs.	15 hrs.	2 hrs.	15 hrs.
Undiluted	+++	+++	±	+++	0	++
1 : 2	+++	+++	++	+++	0	+
1 : 5	+	+++	++	+++	Not set up	
1 : 10	±	+++	0	+++	0	±
1 : 20	0	+++	0	+++	Not set up	
1 : 50	+++	+++	0	+++		
1 : 100	+++	+++	±	+++		
1 : 200	+++	+++	++	+++		
1 : 500	+++	+++	+	+++		
Salt solution (control)	0	0	0	0	0	0

#### DISCUSSION.

The protocols which are reported above are based entirely upon experiments done with macroscopic agglutination carried out in small test-tubes. The sharpness with which these can be read is apparent from figure 1. We have also carried out a number of microscopic agglutinations in which small drops of the mixtures of serum

dilution and treponema emulsion were placed on slides, covered with cover-slips, and rimmed with vaselin. In preparations so made it was very easy to follow the agglutination in a way exactly similar to that habitual in the usual Widal reaction in typhoid fever. The clumping was sharp and distinct, and false clumping, that is, that which would depend upon the presence of disturbing precipitate in the preparations, could easily be ruled out by this method, although our microscopic reactions thus far done were set up with very clean treponema preparations and were interpreted with absolutely no difficulties, as, we think, will be apparent from the photographs attached to this paper (figures 2 to 5).

The results we have obtained, of course, obviously suggest the possibility of working out an agglutination reaction with emulsions of *Treponema pallidum* prepared by our technique, and human sera, with possibly some diagnostic value.

We are especially encouraged in the hope, since Zabolotny and Maslakowetz<sup>s</sup> have observed clumping of the microorganisms in drops of exudate taken directly from primary lesions. In order to obtain some light upon this we immediately carried out a number of microscopic and macroscopic agglutination reactions with sera taken from a series of cases, some of them showing positive and some of them negative Wassermann reactions. It was revealed that many human sera used in concentration and in dilutions as high as 1 to 10 will agglutinate strain A. It will be necessary to examine a large series of normal and syphilitic sera, carefully controlled by clinical observation.

The last experiment we have reported also suggests the possibility that by agglutination it may be possible to determine that a given treponema belongs to the species *pallidum*. However, it will be necessary first to ascertain whether sera prepared by injections of *pallidum* may not also agglutinate spirochetes of other species.

#### SUMMARY.

It has been shown by our experiments that the serum of rabbits treated with emulsions of *Treponema pallidum* contains agglutinating substances.

<sup>s</sup> Zabolotny, D., and Maslakowetz, *Centralbl. f. Bakteriol., 1te Abt., Orig.*, 1907, xliv, 532.



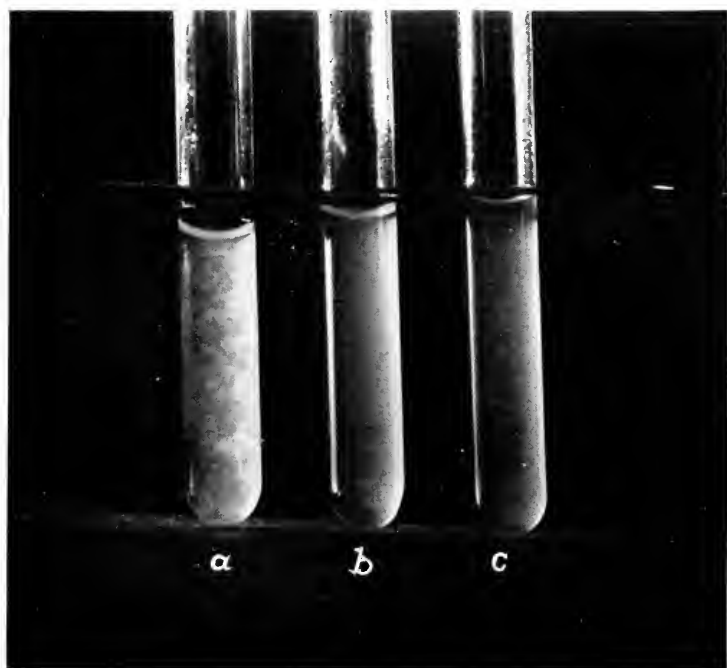


FIG. 1.

(Zinsser and Hopkins: Antibody Formation.)



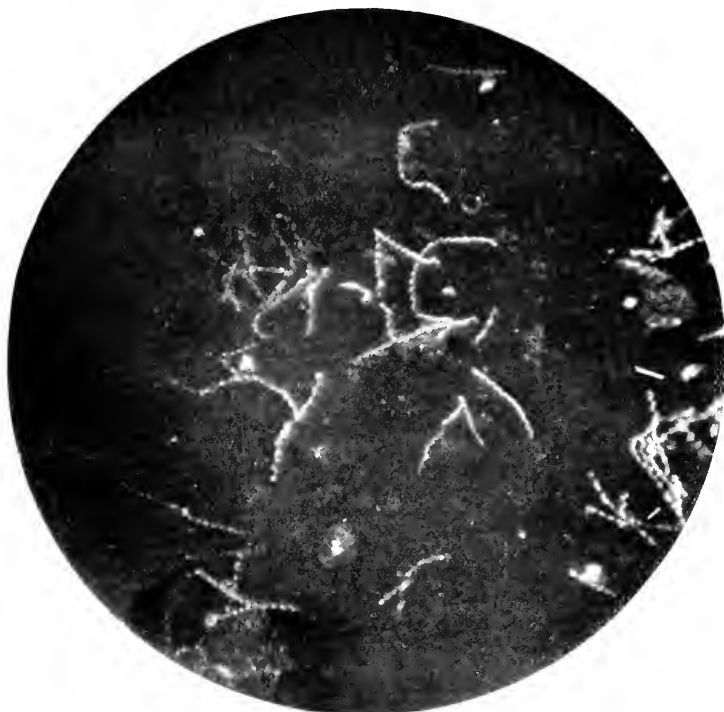


FIG. 2.



FIG. 3.

(Zinsser and Hopkins: Antibody Formation.)



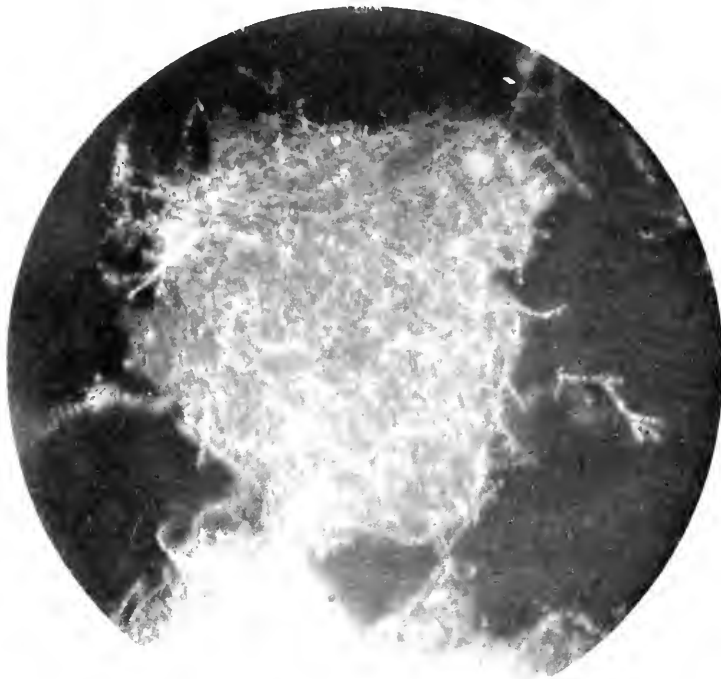


FIG. 4.



FIG. 5.

(Ziesser and Hopkins: Antibody Formation.)



Normal rabbit serum also possesses agglutinating power for this organism, but, as in the case of normal bacterial agglutinins, to an extent very much inferior to that possessed by the sera of immunized animals. Normal human sera will agglutinate similar pallidum emulsions, as will the sera of certain syphilitic patients with positive Wassermann reactions. Whether or not there is a quantitative difference of diagnostic value between the sera of normal human beings and those of syphilitics remains to be seen.

The sera of rabbits immunized with strain A agglutinate Noguchi's strain 9 in dilutions as high as 1 to 500.

We regard as the most important result of these experiments the demonstration of definite antibodies in the circulation of animals treated with dead emulsions of *Treponema pallidum*. Since it is our belief<sup>9</sup> that the agglutinating effect is due to an antibody essentially the same as that which produces bactericidal, precipitating, and opsonic effects, *i. e.*, that there is probably one type of antibody only, we believe that the demonstration of agglutinins establishes the fact that in syphilis as in bacterial diseases the host responds by the formation of antibodies or sensitizers specific for the treponema.

Spirocheticidal experiments with these sera, both *in vitro* and *in vivo*, are in progress.

#### EXPLANATION OF PLATES.<sup>10</sup>

##### PLATE 52.

FIG. 1. Microscopic agglutination. A, immune serum 1:50. B, normal serum 1:50. C, salt solution control.

##### PLATE 53.

FIG. 2. Microscopic appearance of the salt solution control.

FIG. 3. Microscopic appearance of the preparation with normal rabbit serum 1:50.

##### PLATE 54.

FIG. 4. Microscopic appearance of the preparation containing immune serum 1:50.

FIG. 5. Microscopic appearance of the preparation containing immune serum 1:2,000.

<sup>9</sup> Zinsser, H., *Jour. Exper. Med.*, 1913, xviii, 219.

<sup>10</sup> We are indebted to Dr. John E. McWhorter for the photomicrographs reproduced in figures 2 to 5.

ADULT TERTIAN MALARIAL PARASITES ATTACHED  
TO PERIPHERAL CORPUSCULAR MOUNDS. THE  
EXTRACELLULAR RELATION OF THE PARA-  
SITES TO THE RED CORPUSCLES.\*

BY MARY R. LAWSON, M.D.

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PLATES 55 TO 58.

Many of the misconceptions in regard to the morphology and biology of the malarial parasite have risen, I believe, from the fact that the majority of observers have believed the parasite to be intracellular or submerged beneath the surface of the red corpuscle, and that each parasite grew up and completed its life cycle within a single red corpuscle, the segmentation of the parasite corresponding with the final destruction of the red corpuscle.

I have shown in previous publications<sup>1</sup> that the malarial parasite in the course of its development destroys several red corpuscles, migrating from one to another, and that when it is not free in the blood serum, it is attached to the outer surface of the red corpuscle. As Laveran<sup>2</sup> describes it, the parasites are "now free in the blood, now adherent to blood corpuscles."

*The Method by Which the Malarial Parasite Secures Attachment to the Surface of the Red Corpuscle.*—The parasite attaches itself to the outer surface of the red corpuscle by means of pseudopodia encircling a portion of the hemoglobin substance which has been squeezed up by the parasite into a mound with a circular base and a more or less rounded apex. The pseudopodia encircle the mound

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<sup>1</sup> Rowley-Lawson, M., *Arch. Int. Med.*, 1912, ix, 420; *Jour. Exper. Med.*, 1913, xvii, 324; 1914, xix, 450, 523.

<sup>2</sup> Laveran, A., *Paludism*, translated by Martin, J. W., London, 1893, 12.



at its base. The corpuscular mounds may be encircled by a single pseudopodium or by two pseudopodia meeting and fusing.

I believe that the first pseudopodia thrown out arise from the cytoplasm of the parasite near the nucleus. Other (accessory) pseudopodia may be thrown out from any part of the cytoplasm, whenever it becomes necessary for the parasite to secure a firmer hold on the red corpuscle; *i. e.*, when a corpuscular mound to which it is attached becomes dehemoglobinized (decolorized), or as the parasite increases in size.

A very young parasite may find it necessary to use its entire cytoplasm in order to encircle a corpuscular mound, thus giving the appearance, when attached to a surface mound, of a very slender ring with a chromatin dot. When a corpuscular mound encircled by a young parasite is viewed from above, the characteristic ring-form parasite picture is seen.

*Young Parasites Attached to Peripheral Corpuscular Mounds.*—(Figures 1 to 10.) The above interpretation of the ring-form parasite is confirmed by seeing the mounds of corpuscular substance projecting at the periphery of the red corpuscles encircled by the pseudopodia of the parasites.

The encircling pseudopodia may be wholly or in part obscured by the nuclei of the attached parasites; but frequently they may be traced around the peripheral mounds (figures 1 and 10).

Peripheral corpuscular mounds encircled by young parasites are best seen when the corpuscle is newly invaded and the hemoglobin of the mound entirely or nearly intact. As a rule, the corpuscular mounds are rapidly decolorized, proceeding as the parasite digests the hemoglobin. The various stages between the unaltered deeply colored mounds and those which are entirely decolorized may be observed (figures 1 to 10). Rarely the red corpuscle is decolorized before the corpuscular mound.

The decolorized corpuscular mound has been called the "achromatic area" and "milky zone," and many observers have believed it to be an essential part of the parasite.

As additional proof of the extracellular relation of the young parasites to the red corpuscle, the nuclei or the protoplasm may often be seen to project beyond the periphery of the red corpuscle (figures 6, 7, 9, and 10).

*Pigment in Connection with Young Parasites.*—The secretion of pigment by the young tertian parasites seems to follow no known rule. Its first appearance, even in the parasites of a single blood smear, is variable. The decolorization of a corpuscular mound (or even of an entire red corpuscle) may be complete without the appearance of any demonstrable pigment in the young parasite attached to it (figure 6, see parasite at o), and a young parasite without pigment may be of larger growth than a parasite with pigment. And one may see two parasites apparently of equal growth, one with and one without pigment (compare parasites without pigment in figures 1, 3, and 5, with the parasite showing a pigment granule at o in figure 2). And in instances of multiple infection of corpuscular mounds by two young parasites of an apparently equal growth, only one of these parasites may show pigment (figure 7).

This irregularity as to the time when the first pigment appears in the individual young parasite may have no significance other than that certain parasites are slower than others in securing attachment to the red corpuscles, and it may be that some of the parasites are less active than others in the secretion of pigment.

While the presence of pigment in the young parasite has a positive value, its absence does not mean that the parasite has not done its share of injury to the red corpuscle.

*Infection of Corpuscular Mounds by More than One Young Parasite.*—Infection of corpuscular mounds by two or more parasites may be seen (figures 4, 5, x, 6, x, and 7). It has no significance other than accidental occupation. Parasites occupying the one corpuscular mound are usually of one brood and, therefore, in the same stage of development; but parasites of varying ages may also be seen occupying the one corpuscular mound (figure 6, x).

In multiple infections of corpuscular mounds the parasites proceed with the destruction of the mounds in the same way as do single parasites.

*Adult Tertian Parasites Attached to Peripheral Corpuscular Mounds.*—(Figures 11 to 72, and 75 to 108.) While several observers have conceded that the young parasite may be extracellular for a certain time, there has been, with but few exceptions, quite a unanimity of opinion amongst the older writers that the adult para-

site is always intracellular. I have been asked several times if it was possible to demonstrate any appreciable number of adult tertian parasites attached to peripheral corpuscular mounds. These inquiries led to an exhaustive study in search of the same.

Adult parasites are usually seen attached to surface mounds; but peripheral mounds may be overlooked or not recognized, except by the expert observer. Here and there you find adult tertian parasites attached to perfectly recognizable peripheral corpuscular mounds. I believe that adult tertian parasites attached to peripheral corpuscular mounds are only to be found in appreciable numbers in any one smear, soon after a parasitic migration when the infected red corpuscles are not badly damaged; then the peripheral mounds may show more or less of hemoglobin content. But such a condition is so transient that you may have to search for days and examine many specimens before you find what you want.

Corpuscular mounds are quickly dehemoglobinized by adult parasites (figures 50 at 0, 65, 69 to 73, and 105 to 108); I believe more quickly than by the young parasites.

If the tertian malarial parasites were attached to but one red corpuscle during their life time and did not migrate to fresh red corpuscles, I believe that it would be practically impossible to find peripheral corpuscular mounds in connection with the adult parasites.

The adult tertian parasites follow the same procedure in attaching themselves to the red corpuscles as do the young parasites. Primary and accessory pseudopodia encircle surface mounds. As a rule, the pseudopodia which encircle the peripheral corpuscular mounds arise from the cytoplasm of the parasites in the vicinity of the nuclei, as is the case with the young parasites.

The bases of some of the corpuscular mounds in connection with the adult parasites are more constricted than is usual with the mounds seen in connection with the young tertian parasites; *i. e.*, figures 12, 20, 21, and 23. This would indicate greater strength exerted by the adult parasites when encircling the corpuscular mounds.

The nuclei of the adult parasites usually hide from view the encircling pseudopodia, but occasionally the pseudopodia may be seen en-

circling the peripheral mounds (figures 11, 12, 13, 14, 16, 27, 32, 39, 52, 56, 57, 59, 61, 69, 73, 75, 76, 77, 79, 96, 97, 101, 102, and 106).

The nuclei of the adult parasite often extend beyond the periphery of the red corpuscle (figures 12 and 14, 76 and 79), which would not be the case were the parasite intracellular. And some of the adult parasites are so attached to the surface of the red corpuscle that even to the untrained eye they admit of no other interpretation than that they are attached to the outside of the red corpuscle (figures 74 and 109).

*Pigment in Connection with Adult Parasites.*—Adult parasites, apparently in similar stages of development, may show not only a variation in the amount of pigment secreted, but a variation in the size and shape of the granules. Various explanations have been given to account for this, but I shall not enter into a discussion of them in this paper.

*Multiple Infection of Corpuscular Mounds by Adult Parasites.*—More than one adult parasite may be seen to be attached to a single corpuscular mound (figures 18, 19, 44, 63, 72, 86, 87, and 100). As is the case with the young parasites, adults occupying the one mound may be in similar or varying stages of development. Their occupancy of the one mound I believe to be accidental.

I have never found any evidence of the conjugation of malarial parasites. Two parasites attached to one surface mound, with their pseudopodia encircling it superimposed, when viewed from above might give an appearance of conjugating parasites, provided one believed the parasites to be intracellular; but when one knows that they are attached to the one corpuscular mound on the surface of the red corpuscle, then their position in relation to each other is easily explained.

Mounds of hemoglobin substance projecting out at the periphery of the red corpuscles, encircled by the pseudopodia of the two adult parasites, assist in the interpretation of the surface mounds encircled by two adult parasites.

The infected red corpuscles in the pictures which I have selected to illustrate this article show no evidence of crenation or other technical injury.

SUMMARY.

1. The malarial parasite is extracellular throughout its entire life cycle; that is, when it is not free in the blood serum, it is attached to the external surface of the red corpuscle.

2. Adult parasites follow the same procedure in attaching themselves to the outer surface of the red corpuscles as do the young parasites.

3. Adult parasites are most frequently seen attached to surface corpuscular mounds.

4. Corpuscular mounds projecting at the periphery of the red corpuscles and encircled by the pseudopodia of adult parasites, are proof positive of the extracellular relation of the adult parasite to the red corpuscle.

5. Adult parasites attached to peripheral corpuscular mounds are only found in appreciable numbers when the red corpuscles are not badly damaged, so that the mounds show more or less hemoglobin content.

6. The nuclei or protoplasm of adult parasites extending beyond the periphery of the red corpuscles is additional evidence of the extracellular relation of the parasites to the red corpuscle.

EXPLANATION OF PLATES.

PLATE 55.

TERTIAN MALARIAL PARASITES ATTACHED TO PERIPHERAL CORPUSCULAR MOUNDS.

Magnification,  $\times 1,900$ .

FIG. 1. A young parasite attached to a peripheral corpuscular mound. The pseudopodium may be seen encircling the mound.

FIG. 2. A young parasite attached to a peripheral corpuscular mound. A pigment granule may be seen at o.

FIG. 3. A young parasite attached to a partly decolorized corpuscular mound.

FIG. 4. Two young parasites attached to one peripheral corpuscular mound. The nucleus of one parasite partly overlies that of the other parasite, giving the appearance of one large nucleus, but a careful examination will show the presence of two parasites.

FIG. 5. A young parasite attached to a peripheral corpuscular mound and two young parasites attached to a single corpuscular mound on the surface of the red corpuscle.

FIG. 6. Two young parasites, varying somewhat in size, attached to a peripheral corpuscular mound; and at o, a young parasite attached to a completely decolorized peripheral mound.

FIG. 7. Two tiny parasites (it requires the entire cytoplasm of each parasite to encircle the mound) attached to a decolorized peripheral mound. One of the parasites shows a granule of pigment at x.

FIG. 8. A young parasite attached to a peripheral mound which is almost decolorized. A granule of pigment may be seen in connection with the parasite.

FIG. 9. A young parasite attached to a decolorized peripheral mound.

FIG. 10. A young parasite attached to an almost decolorized peripheral mound. A careful examination will show the encircling pseudopodium on the under surface of the mound.

FIG. 11. Two parasites attached to peripheral corpuscular mounds; one of the parasites is much smaller than the other.

FIGS. 12, 13, and 14. Adult parasites attached to peripheral corpuscular mounds. The pseudopodium of each parasite may be seen encircling the mound.

FIGS. 15, 16, and 17. Adult parasites attached to peripheral corpuscular mounds. Figure 15 shows the nucleus of the parasite overlying the encircling pseudopodium. In figure 16 the pseudopodium may be seen on the under surface of the mound. In figure 17 the nucleus of the parasite partly overlies the encircling pseudopodium.

FIGS. 18 and 19. Two adult parasites attached to the one peripheral corpuscular mound.

FIG. 20. An adult parasite attached to a peripheral mound. The mound is somewhat constricted at the base.

FIG. 21. An adult parasite attached to what is really a surface mound, with the periphery of the corpuscle so turned over that the surface mound presents as a peripheral mound. This mound is somewhat constricted at the base.

FIGS. 22, 23, 24, 25, and 26. Adult parasites attached to peripheral corpuscular mounds. Figure 23 shows two peripheral mounds, one of them being very much constricted at its base.

#### PLATE 56.

##### ADULT TERTIAN MALARIAL PARASITES ATTACHED TO PERIPHERAL CORPUSCULAR MOUNDS.

Magnification,  $\times 1,900$ .

FIGS. 27 to 50. Adult parasites attached to peripheral mounds. Figures 29 and 33 show each parasite attached to two peripheral mounds. Figure 50 shows an entirely decolorized mound at o. Figure 44 shows two parasites attached to one peripheral mound.

#### PLATE 57.

##### ADULT TERTIAN MALARIAL PARASITES ATTACHED TO THE EXTERNAL SURFACE OF THE RED CORPUSCLES.

Magnification,  $\times 1,900$ .

FIGS. 51 to 59. Adult parasites attached to peripheral mounds.

FIG. 60. An adult parasite attached to what is really a surface mound. The periphery of the corpuscle is turned over so that the surface mound presents as a peripheral mound.

FIGS. 61 and 62. Adult parasites attached to partially decolorized peripheral mounds.

FIG. 63. Three parasites attached to one peripheral mound. The mound is much decolorized.

FIG. 64. An adult parasite attached to a partially decolorized peripheral mound.

FIG. 65. An adult parasite attached to a decolorized corpuscular mound.

FIG. 66. An adult parasite attached to a peripheral mound. The mound shows a partial decolorization.

FIGS. 67 and 68. Adult parasites attached to peripheral corpuscular mounds which have been partly dehemoglobinized by the action of the parasites.

FIGS. 69, 70, and 71. Adult parasites attached to completely decolorized corpuscular mounds.

FIG. 72. Two adult parasites attached to a completely decolorized corpuscular mound.

FIG. 73. An adult parasite attached to a decolorized corpuscular mound.

FIG. 74. An adult parasite attached to the surface of the red corpuscle in such a manner that even the most skeptical observer would not think it to be other than extracellular.

#### PLATE 58.

COLORING PHOTOGRAPHS OF THE PARASITES SHOWN IN PLATES 55, 56, AND 57.

Magnification,  $\times 1,900$ .

FIG. 75. (Corresponds to figure 11.) Two parasites in varying stages of development attached to peripheral corpuscular mounds.

FIGS. 76, 77, 78, and 79. (Correspond to figures 12, 13, 28, and 14.) Adult parasites attached to peripheral corpuscular mounds. The pseudopodia of the parasites in figures 76, 77, and 79 may be seen encircling the mounds.

FIG. 80. (Corresponds to figure 15.) Adult parasite attached to a peripheral mound. The nucleus of the parasite overlies the encircling pseudopodium.

FIG. 81. (Corresponds to figure 16.) An adult parasite attached to a peripheral mound. The pseudopodium may be seen encircling the mound.

FIGS. 82, 83, and 84. (Correspond to figures 17, 20, and 22.) Adult parasites attached to peripheral mounds. Figure 83 shows the base of the mound to which the parasite is attached to be slightly constricted.

FIG. 85. (Corresponds to figure 21.) An adult parasite attached to what is really a surface mound. The periphery of the red corpuscle is turned over so that the surface mound presents as a peripheral mound.

FIGS. 86 and 87. (Correspond to figures 18 and 19.) Two adult parasites attached to one corpuscular mound.

FIGS. 88, 89, and 90. (Correspond to figures 24, 25, and 47.) Adult parasites attached to peripheral corpuscular mounds.

FIG. 91. (Corresponds to figure 29.) An adult parasite attached to two peripheral corpuscular mounds.

FIGS. 92, 93, and 94. (Correspond to figures 31, 34, and 35.) Adult parasites attached to peripheral corpuscular mounds.

FIG. 95. (Corresponds to figure 23.) An adult parasite attached to two peripheral corpuscular mounds. One of the mounds is very much constricted at its base.

FIGS. 96, 97, 98, and 99. (Correspond to figures 39, 52, 41, and 42.) Adult parasites attached to peripheral corpuscular mounds.

FIG. 100. (Corresponds to figure 63.) Three parasites attached to one corpuscular mound.

FIGS. 101, 102, 103, and 104. (Correspond to figures 37, 61, 62, and 40.) Adult parasites attached to more or less decolorized corpuscular mounds.

FIGS. 105, 106, 107, and 108. (Correspond to figures 50, 69, 70, and 71.) Adult parasites attached to decolorized corpuscular mounds.

FIG. 109. (Corresponds to figure 74.) An adult parasite attached to the external surface of the red corpuscle.





(Lawson: Adult Tertian Malaria Parasites.)





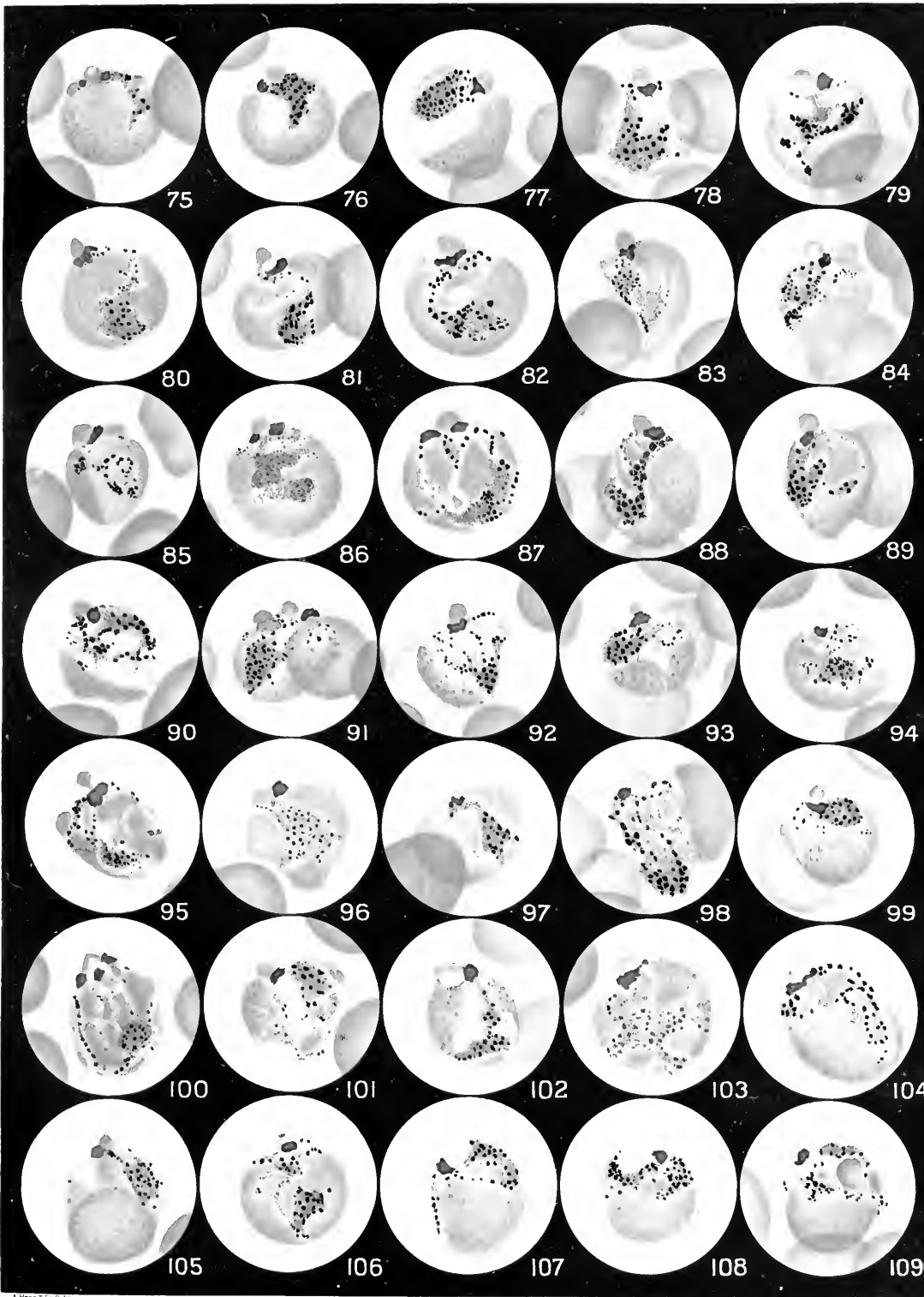
(Lawson: Adult Tertian Malarial Parasites.)





(Lawson: Adult Tertian Malarial Parasites.)





A. H. H. 5' 10 100

(Lawson: Adult Tertian Malarial Parasites.)





## THE INFLUENCE OF DIGITALIS ON THE T WAVE OF THE HUMAN ELECTROCARDIOGRAM.\*

By ALFRED E. COHN, M.D., FRANCIS R. FRASER, M.D., AND  
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PLATES 59 TO 64.

The modifications in the form of the T wave of the electrocardiogram which we report in this communication were observed in the course of detailed studies on the action of digitalis in patients (1). It has been noticed frequently that the time when digitalis takes effect is a matter of uncertainty, so that in a given instance doubt is often entertained as to whether the drug itself is active, and if active, whether it is effective in producing a result. To be certain that the drug is effective, it has been considered necessary to administer it until an alteration in the rhythm of the heart occurs. In our treatment of patients we have, therefore, looked for evidences of altered heart rhythm to indicate that the heart is really digitalized. During the period of treatment, electrocardiograms were made almost daily, and often more than once a day. In examining these records we found the changes in the T wave which we describe in this report.

Other observers have published studies dealing with the effect of a number of agents, including digitalis, on the electrocardiogram. The only investigation dealing with this subject in human beings has been made by Nicolai and Simons (2). Seven ambulatory patients were given powdered digitalis, 0.3 gm. daily, for five days. In two women the treatment was not completed; in one normal man the T wave did not increase in height, but decreased instead (*"verschlechterte sich"*); and in the other four the T wave increased. They limited the taking of records to the first lead (right arm to left arm). Even if the

\* A preliminary report was made to the Section on Medicine at the Seventeenth International Congress of Medicine, London, 1913, Section VI, 255-258. Received for publication, April 7, 1915.

dosage had been sufficient, and had been satisfactorily controlled, a comparison of their methods with ours would account for the difference in our results.

A number of experiments on animals have been published in which digitalis bodies, more especially strophanthin, were injected for the purpose of observing possible changes in the electrocardiogram under the influence of the drug. Straub, Selenin, Bickel and Tsividis, Bickel and Pawlow, and Rothberger and Winterberg have published such reports. In general all observers have detected an increase in size of the T wave under the influence of digitalis, like that described by Nicolai and Simons. Straub (3) described experiments in cats and rabbits, into the veins of which he injected strophanthin (Böhringer). So called therapeutic doses, the exact quantity of which is not given, caused no alterations in their electrocardiograms. When he gave toxic doses, that is to say, 0.3 mg., he obtained an increase in the elevation of the T waves in one experiment, the curve of which he reproduces. He obtained a multiplicity of abnormal ventricular complexes in the late stages of intoxication, but obviously the T waves in these forms cannot be compared with those in the control curves. In a later paper (4) he described experiments on the isolated frog heart, and showed that in the therapeutic stage the T wave increased in size, as in his mammalian experiments, while in the late toxic stages the T wave became negative. The changes in the late toxic stage in frogs are not like those in mammals, because the former do not develop abnormal ventricular complexes.

Selenin (5) found that the T wave increased in size when a digitalis body was given in therapeutic doses to dogs.

Bickel and Tsividis (6) injected digitalysatum Bürger into the ear veins of rabbits. Small and medium doses (1 c.c. per kg.) increased the size of the waves. Larger doses decreased the size of the waves, including the T wave, but they remained upward in direction.

In a later paper Bickel and Pawlow (7) injected crystalline strophanthin (Thoms) intravenously into dogs, and digistrophan, a combination of digitalis and strophanthin, into dogs and rabbits. They concluded that small doses increased the size of the T wave, whereas large doses decreased the size of all waves. They mention no instance of inversion. They found that the T wave tended to change less than the other waves. Other substances supposedly effectual on the heart, like cardiotoxin, a mixture of convallaria and caffein sodium benzoate, were quite ineffectual.

Rothberger and Winterberg (8) found that small doses of strophanthin given to dogs had no effect on the shape of the electrocardiogram. When P and T waves were small as the result of the heart's having been isolated from the central nervous system, they report that digitalis restored them to their initial size. When they gave digitalis in progressively larger quantities, aside from alterations in the P, R, and S waves, the T wave became either positive or negative. In the experiments of all these investigators the results depend on the injection of single doses of the drug, the quantities being larger than those which are employed in therapeutics. The change in the electrocardiogram usually obtained is considered to be an increase in the elevation of the T wave. Straub alone seems to have regarded the change in sign in the T wave in his experiments on frogs as unusual and important.

The shape of the T wave has also been changed by the use of other means.

Muscarin, according to Samojloff (9, 10, 11) and Boruttau (12), alters not only its form, but in frogs changes the sign of the wave from positive to negative. After the alteration has taken place, both noticed that the application of atropin restores the initial direction of the wave. Mines (13) believed that spread from the site of application to the ventricular base was responsible for Samojloff's result and that muscarin does not alter the sign of the T wave except on direct application. After alteration has taken place, atropin relieves it. But in his figures 2 and 3 the sign of T changed from negative to positive. Here the heart was perfused with muscarin so that the possibility of local action is excluded. Samojloff and Mines (14, 15) both observed that electrical stimulation alone of the vagus nerve in frogs without the use of drugs produced an inversion of the T wave.

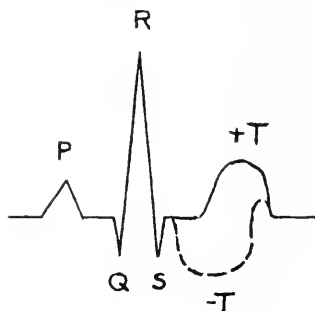
#### OBSERVATIONS.

In the curves of the patients under treatment with digitalis, attention was arrested by the fact that alterations in the T waves of the electrocardiograms occurred in a large number (30 times in 34 patients), and also by the fact that the change was detected before alterations in rhythm or conduction time had occurred and before symptoms referable to the gastro-intestinal tract disturbed the patients, except in five instances. The changes in the T wave consisted in a diminution in the height of the wave, and, finally, in an inversion (figure 1). This diminution in height to an isoelectric line and subsequent inversion of the wave are not the only changes which occurred. If the space from the end of R or S to the end of T is considered, additional alterations become evident. The first part of this interval, from R or S to T, is usually isoelectric or shows a slight upward slope. In place of these conditions may be substituted either an isoelectric period, if it is not isoelectric already, or a downward deflection, directly continuous with the end of R or S. The downward deflection is often carried to a point just short of where in the uninfluenced curve the T wave ends. At this time, then, the inverted wave passes above the base line to form an upward deflection, representing the terminal portion of the original wave. It is indicated in figure 1 D. Text-figure 1 shows this relation. The solid line indicates the outline of the initial curve; the dotted line, the alteration described. Samojloff (11) has drawn a similar diagram.<sup>1</sup> Another alteration which is sometimes seen is shown in text-figure 2. Here the downward deflection occurs, not in the

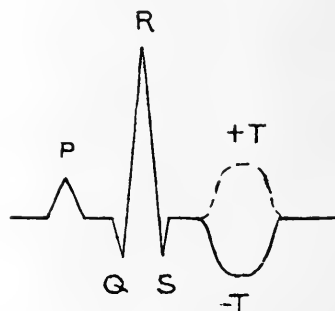
<sup>1</sup> Samojloff, *loc. cit.* (11), p. 481, figure 2 C.

early portion of the period, but in the later portion. Instead of a diphasic wave in which the upward deflection occupies the last portion, as in text-figure 1, it is in such instances in the first portion.

Other alterations beside those described have been seen when digitalis was administered. One was in a case of complete auriculo-ventricular dissociation. The observation in this patient was especially valuable because he showed, at different times, two quite differ-



TEXT-FIG. 1.



TEXT-FIG. 2.

TEXT-FIGS. 1 and 2. The unbroken line represents the electrocardiogram, and shows the P, Q, R, S, and T waves. The broken line shows the outline the T wave assumes under the influence of digitalis.

ent electrocardiograms. One indicated an origin for the ventricular beats in the left side of the heart (figure 2); the other, in the right side (figure 3). In figure 2, taken when the ventricular beats were initiated on the left side, inverted  $T_1$ <sup>2</sup> became deeper under the influence of digitalis, inverted  $T_2$  moved upward, and so did diphasic  $T_3$ . When the ventricular beats were initiated on the right side (figure 3) diphasic  $T_1$  became isoelectric, inverted  $T_2$  turned upward, and inverted  $T_3$  became shallow, almost isoelectric, and diphasic, while the completely restored curve resembled its control except that  $T_1$  was a monophasic wave, replacing a diphasic wave in the initial curve. That the change was less far reaching in figure 3 than in figure 2 may have been due to the lesser amount of digitalis which had been given, 1.9 instead of 2.6 grams. These curves supply an instance of a change in the sign of  $T_2$  and  $T_3$  from negative to posi-

<sup>2</sup>  $T_1$  represents the T wave in lead 1;  $T_2$  in lead 2, etc.

tive under the influence of the drug, a change of sign in the reverse direction from that first described.

Still other types of change have been found. In a case, for instance, in which  $T_1$  and  $T_2$  were positive, and  $T_3$  negative (figure 4),  $T_2$  became negative, and  $T_3$  more negative. In pathological hearts, such as these, the redistribution of muscle, resulting from hypertrophy and dilatation, doubtless determines a variety of alterations in the electrocardiogram under the influence of digitalis. We do not at the present time possess a sufficient number of instances of a given class to permit us to reduce the alterations in them to definite order. Originally it appeared to us that  $T_3$  altered earlier than  $T_2$ . Our later observations make us hesitate to state definitely whether the T wave in one lead is influenced earlier than in the others, or whether changes occur simultaneously in all. Recent interpretations of the electrocardiogram by Einthoven, Fahr, and de Waart show that simultaneous alterations may be anticipated, and our later observations indicate that this is so.

The instances so far described relate to cases in which the mechanism of the heart beat was normal. Similar alterations have, however, also been seen when the auricles were fibrillating (figure 5) and also when they fluttered. In curves of this type, recognition of altered T waves may occasionally be difficult, especially if the waves are not large, on account of the greater number of oscillations in the curve, due to the abnormal auricular activity.

We have pointed out that with five exceptions the modifications described were seen at a time when neither a change in rhythm or conduction time nor the onset of gastro-intestinal symptoms had taken place. An alteration in the curve may, therefore, be taken as a sign of the fact that an influence by digitalis is being exerted on the heart. The sign attains the greater importance on account of its appearance early after the beginning of the administration of the drug. We have detected changes in the T waves after an equivalent of 1.2 grams or even less of the dried leaves<sup>3</sup> of digitalis has been given, that is to say, on the third day of administration, although

<sup>3</sup> We have used digipuratum, a tablet of which represents 0.1 gm. of dried leaves. We usually give 0.4 gm. a day. The dose varies naturally with the patient's requirements. We have not estimated the size of the smallest dose capable of producing a change.

on several occasions we have seen the altered forms after thirty-six to forty-eight hours. On account of certain matters of technique, we have hesitated, in our earlier records, to recognize the onset of the alteration as early as it is quite apparent to us now that it occurs. The sign may, therefore, be looked on as one capable of detection earlier than the others, and as one indicating the fact that the drug is acting. Small changes measured in hundredths of seconds in the auriculoventricular conduction time occasionally occur as early as that noticed in the T wave, and when present may, of course, be employed as corroborating evidence of the fact that the drug is acting. The length of time required before the T wave is restored to its initial state varies. In the patient whose curves are reproduced in figure 6 twenty-two days elapsed before complete restoration occurred. In other cases it required only five days.

The influence of atropin on the T wave, after an alteration in it has become established, was tested more than forty times. Atropin sulphate was given either subcutaneously or intravenously in doses ranging from 0.9 to 1.8 milligrams, the size of the dose depending on the age of the patient and the method of administration. In every instance, the alteration which had taken place persisted after the drug was given. In several instances, when no change had been observed before atropin was injected, the characteristic modification appeared under the influence of the drug. Those alterations which atropin brought on or intensified almost always disappeared promptly; that is to say, before the next curve was taken, twenty-four hours later. Atropin alone, when no digitalis had been given, did not produce changes in the T wave. We have, however, an instance in which atropin lowered the isoelectric line between R and T slightly. Another modification in the outline of the digitalis curve occurs during the activity of atropin. The acceleration in rate which atropin causes is accompanied by a reduction in the time which elapses from the beginning of R (Q) to the end of T (figures 1 and 6 and text-figures 1 and 2). As a result of acceleration, the various elements which make up the ventricular electrocardiogram contract, but the essentials of the change which digitalis has brought about in the curve remain quite unaffected.

## DISCUSSION.

In view of the fact that for human beings the form of the electrocardiogram is constant, and varies only because of distinct cause, the changes in it which have just been described must be looked upon as changes of significance. That they result from the action of digitalis must be concluded from the fact that they occur almost invariably when the drug is administered, and disappear more or less promptly when administration of the drug is discontinued. If the drug is given again to the same patient, after an interval during which the initial form of the electrocardiogram is restored, the same changes take place.

The interpretation of the changes in the T wave which have been described as occurring under the influence of digitalis presents difficulties, based on the interpretation of the formation of the electrocardiogram itself. If we adopt the current view, held by the earlier writers, Burdon-Sanderson and Page, Bayliss and Starling, and most of the later investigators, Einthoven, Samojloff, Garten, Boruttau, and Mines, that the cardiac action currents depend on electrical changes as an expression of muscular activity, then the changes in the T wave must be attributed to an alteration in muscular state under the influence of the drug. The fact that the T wave becomes negative indicates that the preponderance of electrical activity persists in the region near the apex of the heart longer than it does at the base; or, conversely, shorter at the base than at the apex. This is substantially the explanation that Samojloff and Boruttau adopt in explaining the action of vagus excitation and of muscarin. It opposes the view that persistence of electrical activity depends on a specific action directed toward a given part of the heart muscle, as Selenin supposed.<sup>4</sup> Greater electrical activity near the apex, therefore, accounts for apex negativity, and the consequent inversion of the T wave. The variations in the alteration of the terminal part of the wave already described depend on the exact site of longer electrical activity. If electrical activity at the base outlasts that at the apex, a diphasic wave results, the first portion of which is directed downward, corresponding to greater activity at the apex, and the second phase ends in a short upward deflection, corresponding to

<sup>4</sup> Selenin, *loc. cit.*, p. 154.

greater persistence of activity at the base (text-figure 1). Occasionally activity lasts longer, in the terminal phase of ventricular systole, at the apex, and this is shown by a diphasic wave, the direction of which in its first portion is upward, if the activity at the base is the greater, and in the latter portion downward (text-figure 2).

The observations of numerous pharmacologists on the action of digitalis bodies on the frog's and on mammalian hearts have demonstrated a diminished diastolic relaxation of the apical regions of the ventricles. In the later toxic stages of the action of the drug a systolic standstill of the apical regions is seen, while the base is still relaxing in diastole.<sup>5</sup> The changes we have observed in the T wave during the action of digitalis on the human heart may be evidence of a similar condition of the apical portion of the ventricles.

A fact to which we have called attention in these observations necessitates a possible modification in the view that the effect of digitalis is, so far as its influence on the T wave is concerned, wholly muscular. We refer to the fact that, on occasion, when a heart is subjected to the influence of digitalis, no change or only a slight one, takes place in the outline of the T wave. If atropin is injected under these circumstances, the alteration in the T wave, which was expected but failed to appear, becomes manifest immediately; the T wave either becomes inverted or becomes more negative than it was. When the influence of atropin terminates, the T wave returns to the state before injection. The view taken that atropin acts on the terminals of the inhibitory nerves implies, in this connection, that these exert an influence on the alteration in the wave, besides that attributable to the action of digitalis on heart muscle. Their influence, stimulated perhaps by the action of digitalis, is exerted against the full expression of increased electrical activity on the part of a given muscle area. When atropin is given, the inhibitor power is removed, and the full influence of digitalis on the muscle is manifest.

<sup>5</sup> This phenomenon is recorded by Cushny, A. R., *A Textbook of Pharmacology and Therapeutics, or the Action of Drugs in Health and Disease*, 4th edition, Philadelphia, 1906, 439. Asynchronous contractions of the frog's ventricle, due to strophanthus, are also described by Fraser, T. R., *On the Kombé Arrow-Poison (Strophanthus cuspidus, D.C.) of Africa*, *Jour. Anat. and Physiol.*, 1873, vii, 139.



These observations have yielded two-fold evidence bearing on the nature of the mechanism of digitalis action, considered from the point of view of whether this is muscular or nervous. We recall, in the first place, the difference between the effect of atropin on the T wave during digitalis, and during muscarin action. It has been shown that both drugs are capable of producing modifications in the T wave. The injection of atropin in the case of muscarin abolishes these, according to Samojloff and Boruttau, while in the case of digitalis the change persists or is intensified. The difference brought out between the two by the injection of atropin appears to depend on a difference in the site of their respective actions. The site of the action of muscarin on the cardiac inhibitors is well established;<sup>6</sup> its effect is consequently abolished when atropin, which acts on the nerve terminals, is given. It follows from this that if the inversion of the T wave under the influence of digitalis is not relieved by the injection of atropin, that influence is exerted at a site distal to the nerve endings; that is to say, on the heart muscle. We have shown that the inhibitors can prevent the formation or the full development of inversion in the T wave because it sometimes does not appear until after atropin is given. This curious phenomenon is exerted in opposition to the obvious muscular digitalis effect, and represents what appears to be a new phase of the function of the nerves. The second observation relating to the mechanism of the action of digitalis to which we have referred shows that it has two separate and distinct effects on the heart. The first consists in an alteration in the mechanism of the heart beat, a function which has been abundantly proved, and the second in the alteration of the T wave we are describing. The effect of the injection of atropin indicates that these two alterations depend on two activities of digitalis, quite distinct from each other, for the altered mechanism is abolished, while the altered T wave either persists or is intensified (figure 6). We attribute the altered heart rhythm which atropin is able to relieve to the action of digitalis on the inhibitory mechanism. The altered T wave which atropin fails to restore to its initial

<sup>6</sup> Recently the site of the action of muscarin has been reconsidered in papers by Straub, Samojloff, and Mines. Inasmuch as all agree that atropin has the effect mentioned, the argument advanced is considered valid.

state we attribute, as we have just shown, to the muscular action of digitalis. We assume for the purpose of this discussion that the effect of atropin is uniform; but we shall show in another place that this is true only within certain limits.

We wish finally to indicate the bearing that these observations have on the phenomenon of persistence of digitalis action. If alteration of the T wave depends on the action of digitalis, the time which elapses between the end of its administration and the return of the T wave to its initial state must be considered the measure of time during which digitalis is an active agent. The length of time required for restoration to the initial state has been seen to vary. In one observation it has been seen to take as little as five days; it has been observed to require as long as twenty-two days. The latter, if it is a common example of the duration of action, is a longer period for the drug to be active than is commonly supposed. Persistence of action may be compared with the duration of digitalis effect on rate in auricular fibrillation. In at least one such patient we have several times seen fourteen days elapse, after the administration of digitalis was stopped, before the ventricular rate began to rise. The statement (16) sometimes made that a second course of treatment with digitalis requires less of the drug to produce a given effect than the first is probably explained by our observation. For it is clear that if a second course is begun, before the first is completely terminated, a greater amount, equal to the residual quantity of the drug, must be present in the heart than had been expected.

#### SUMMARY.

It has been shown in this investigation that digitalis, administered orally to patients, can modify the T wave in the electrocardiogram. When the T wave in the initial curve is directed upward, the first change noticed is a lowering, and the final change is an inversion of the wave. It is not only the wave itself, but that portion of the curve between the end of R and the end of T which is involved. Instances in which the initial T waves have other than upright forms are described and their behavior under the influence of digitalis has been indicated. This influence of digitalis on the T wave may be

detected in thirty-six to forty-eight hours after the administration of digitalis has commenced; it may persist as long as twenty-two days after the administration has been stopped. Instances where it persisted only five days have been encountered. The unexpected length of duration of the sign probably explains why a second treatment with digitalis requires a smaller amount of the drug to produce the same effect, than the first.

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14. Mines, On Functional Analyses by the Action of Electrolytes, *Jour. Physiol.*, 1913, xlvi, 188.
15. Mines, Further Experiments on the Action of the Vagus on the Electrogram of the Frog's Heart, *Jour. Physiol.*, 1913-14, xlvi, 419.
16. Eggleston, C., Clinical Observations on the Duration of Digitalis Action, *Jour. Am. Med. Assn.*, 1912, lix, 1352.

## EXPLANATION OF PLATES.

In all figures divisions of the abscissæ equal 0.04 of a second; divisions of the ordinates equal  $10^{-4}$  millivolts.

## PLATE 59.

FIG. 1. A to H. The curves were taken from a patient who had a moderate grade of arteriosclerosis. A is the control. B, C, D, and E were made under the influence of digitalis; a few minutes before E atropin was injected. F and G show the gradual return to the form of the control. H substantially reproduces the control, A. The date to the left of each curve indicates when the curve was taken. The three usual leads (Einthoven) arranged in columns were obtained on each occasion. During the atropin test only lead 2 was made. The total amount of digitalis taken at the time each curve was made is given at the right.

## PLATE 60.

FIG. 2. A to C. The curves were taken from a patient who had complete auriculoventricular dissociation. The three leads are arranged from above down. A is the control. B was taken after digitalis, 2.6 gm., was given. C substantially reproduces the control. The date on which each set of three curves was made is placed below, likewise the rate of the ventricles.

## PLATE 61.

FIG. 3. A to D. These curves were taken from the same patient as those in figure 2. A is the control; B was taken under the influence of digitalis; C shows partial recovery; D substantially reproduces the form of the control curve.

## PLATE 62.

FIG. 4. A to L. These curves were taken from a patient who had mitral stenosis. The leads are arranged in columns. The date when each curve was taken is given at the left. A is the control. B, C, and D were taken under the influence of digitalis; the amount taken when the curve was made is given at the right. E to K shows the gradual return to the control. J, a single instance, breaks the gradation. L substantially reproduces the control curve.

## PLATE 63.

FIG. 5. A to C. These curves were taken from a patient whose auricles were fibrillating. The leads are arranged as in figure 2. A was taken after digitalis, 3.2 gm., had been given. B is the control. C was taken after digitalis, 3.6 gm., had been given.

## PLATE 64.

FIG. 6. A to C. These curves were taken from a patient who has mitral stenosis. The leads are arranged as in figure 2. A is the control. B was taken after digitalis, 2.5 gm., was given. The altered cardiac mechanism is described in the text. B. was made shortly after B. when atropin, 1.0 mg., had been injected. C substantially reproduces the control curve.

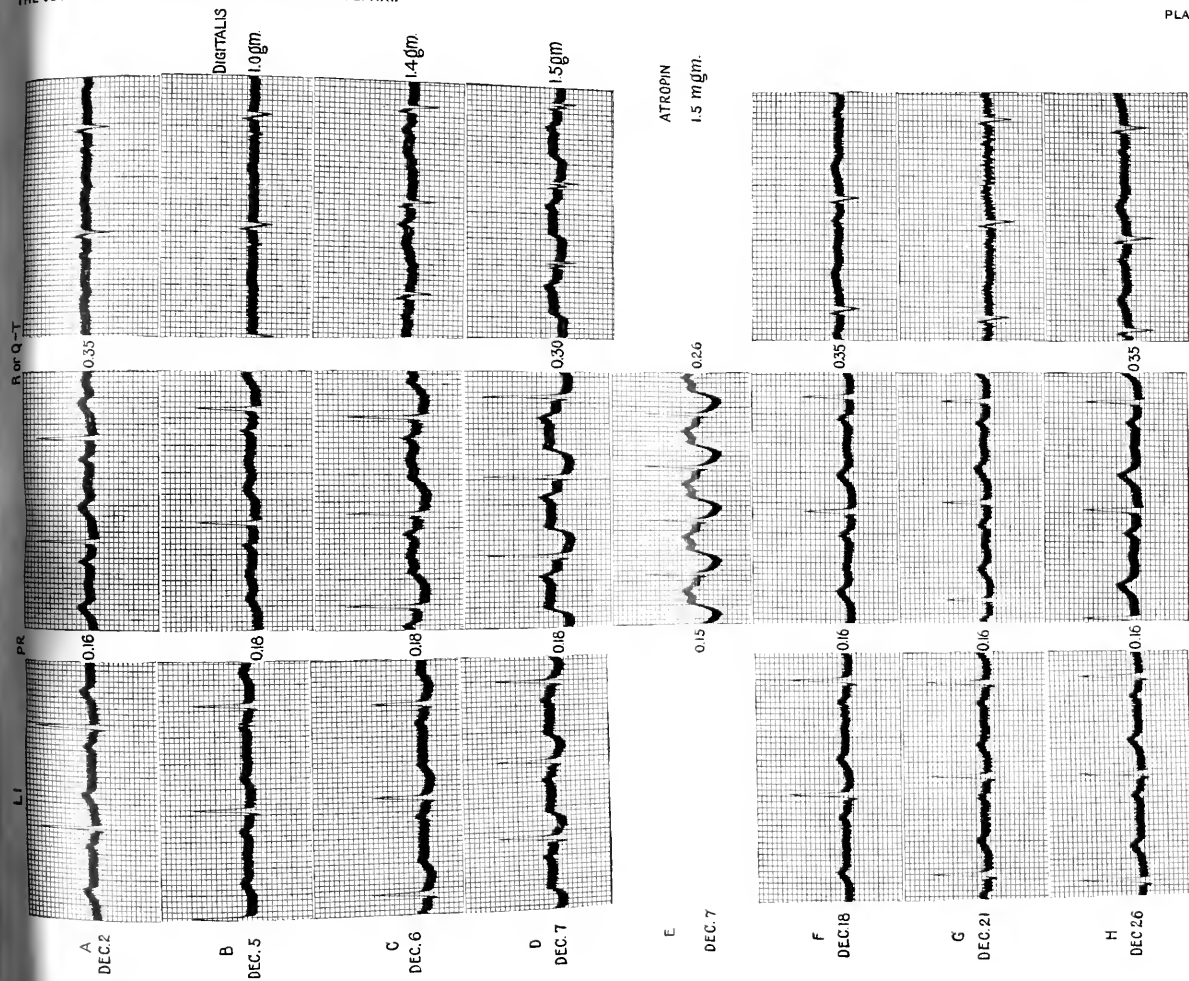
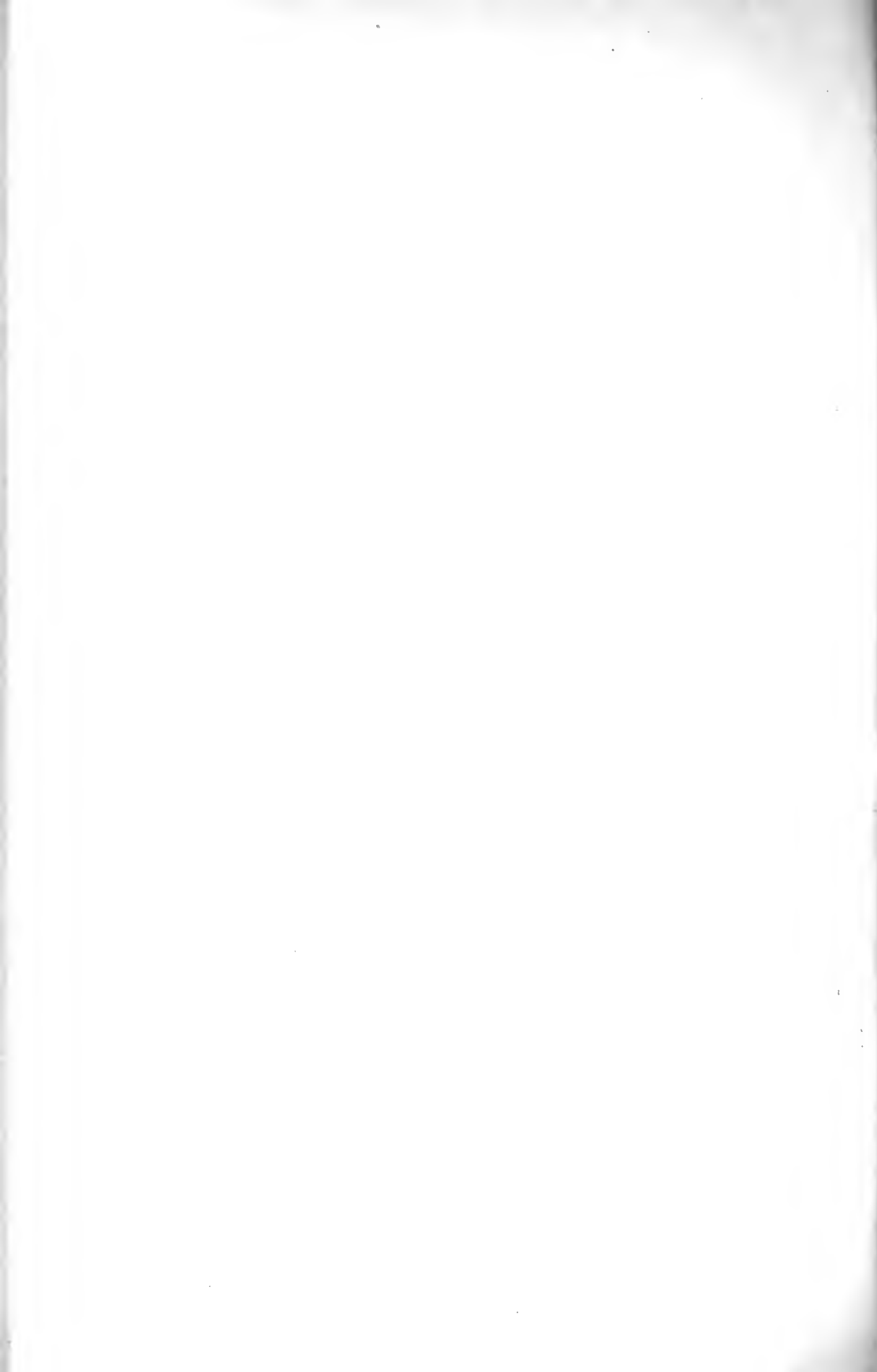


FIG. 1.  
(Cohn, Fraser, and Jamieson: Influence of Digitalis on Human Electrocardiogram.)



604<sup>2</sup>

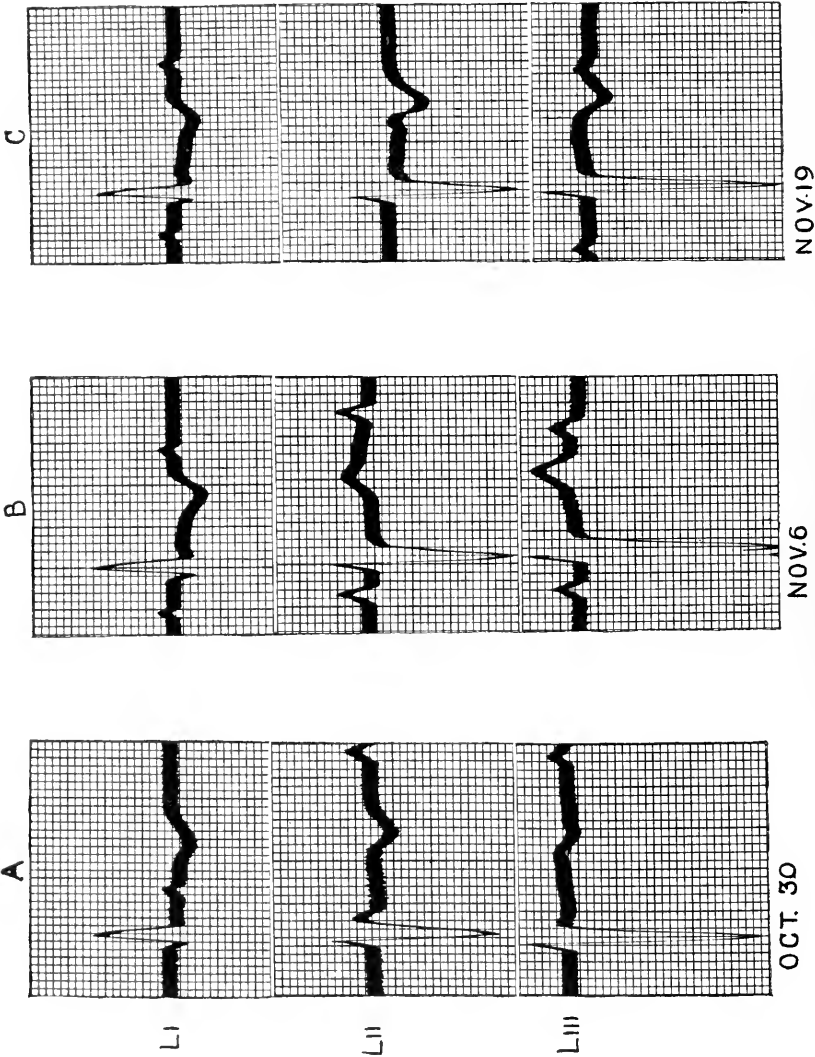


FIG. 2.

(Cohn, Fraser, and Jamieson: Influence of Digitalis on Human Electrocardiogram.)



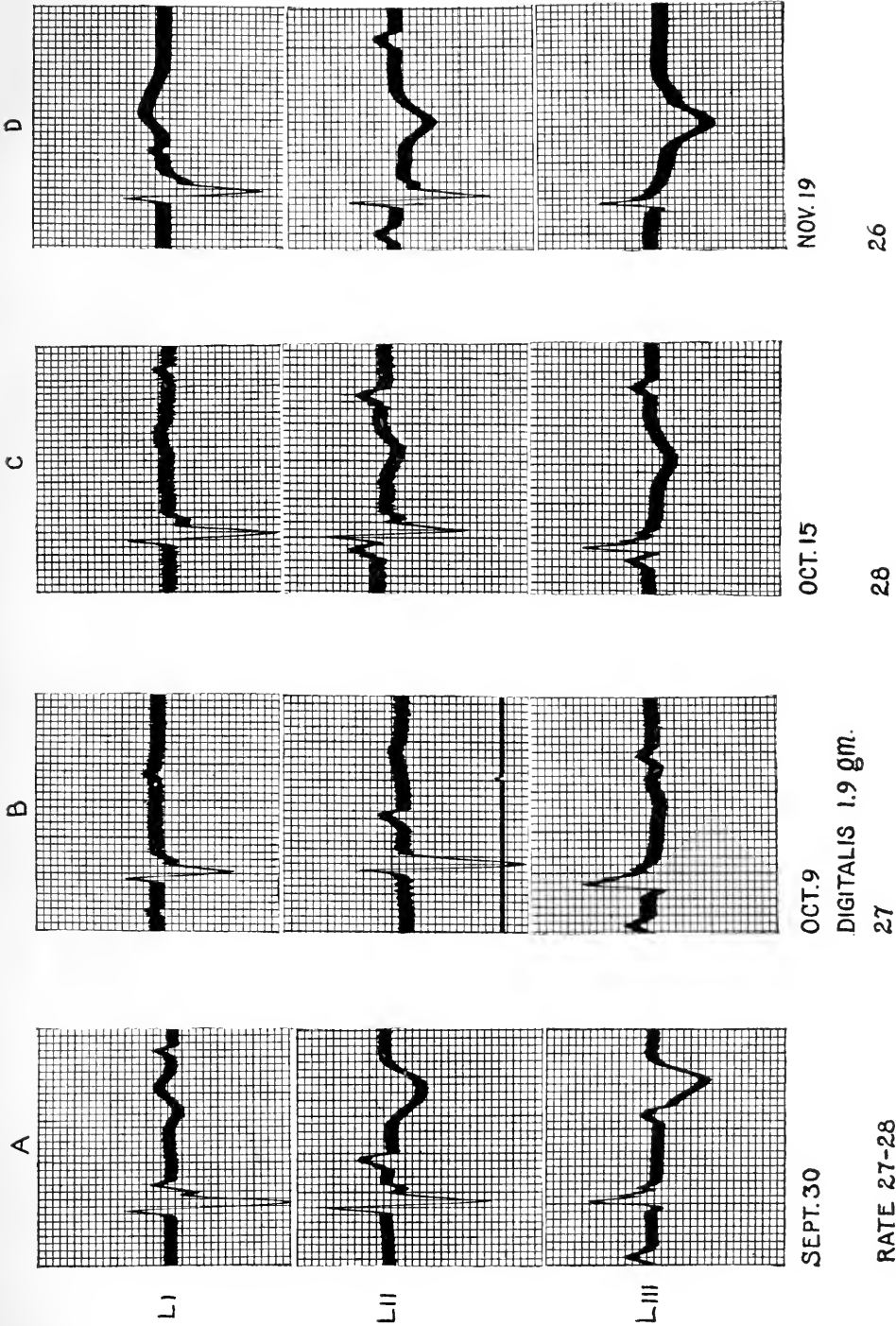


FIG. 3.  
(Cohn, Fraser, and Jamieson: Influence of Digitalis on Human Electrocardiogram.)



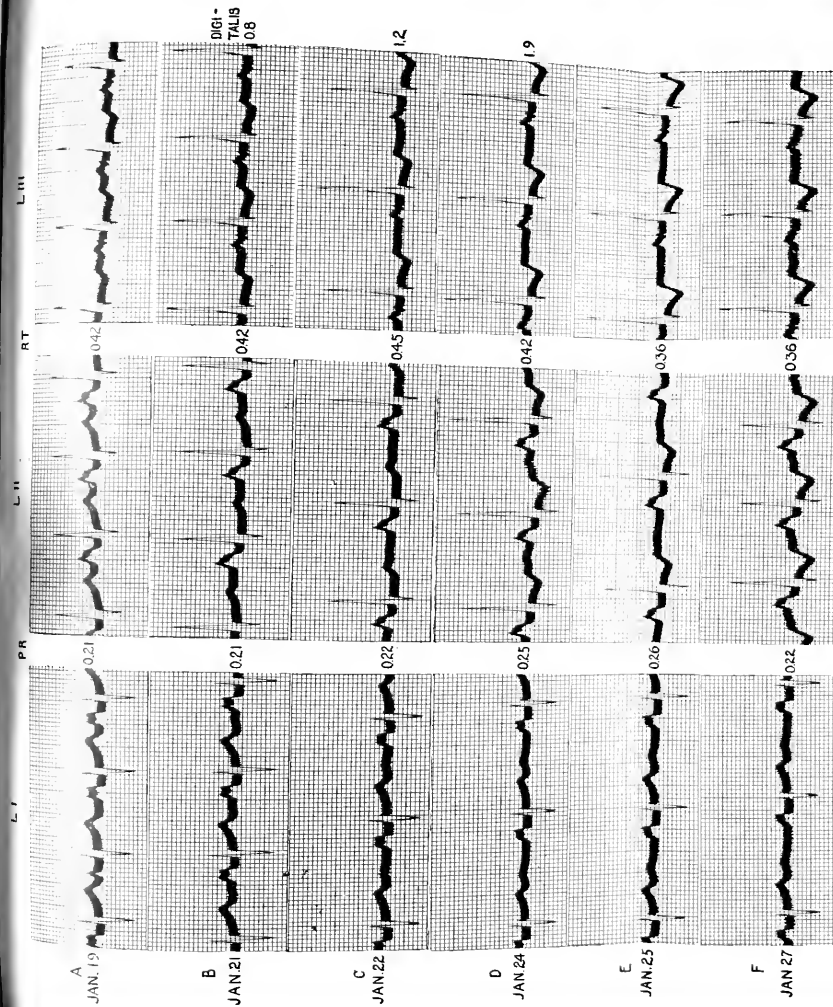


FIG. 4.

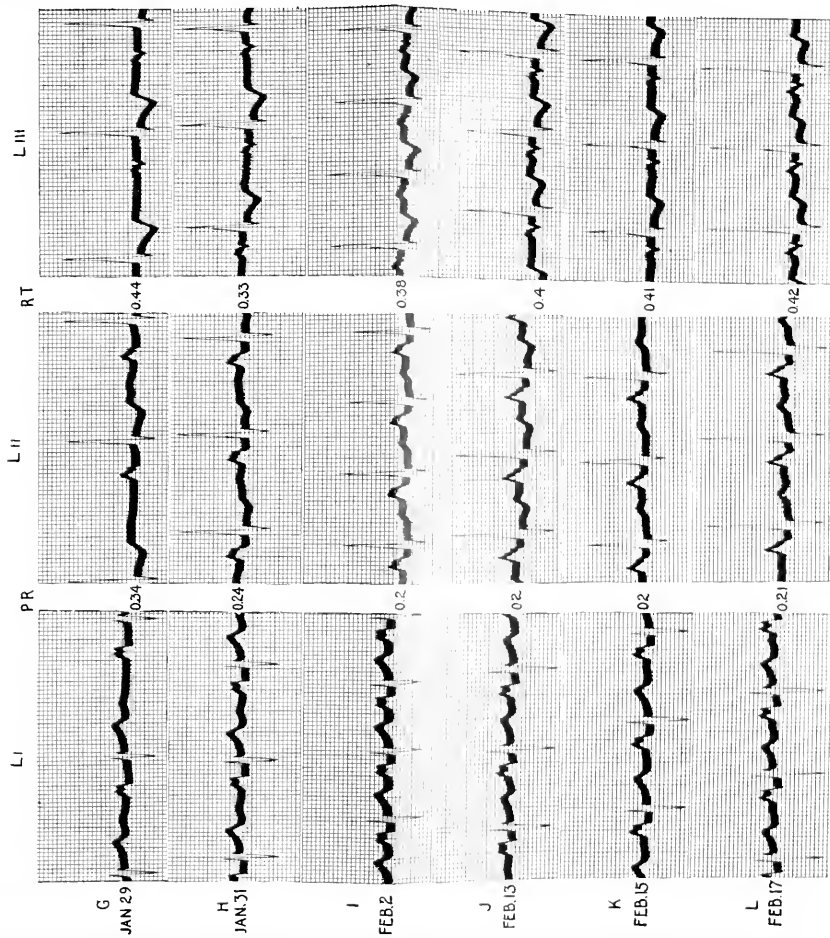
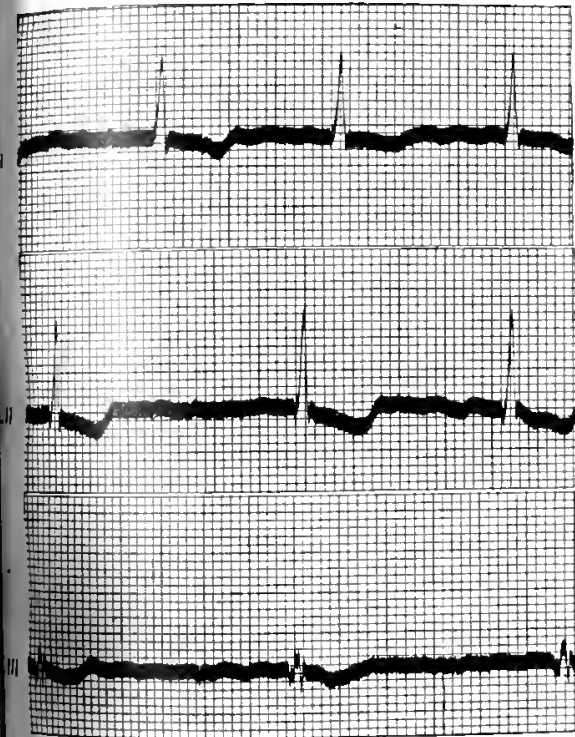


FIG. 4A.

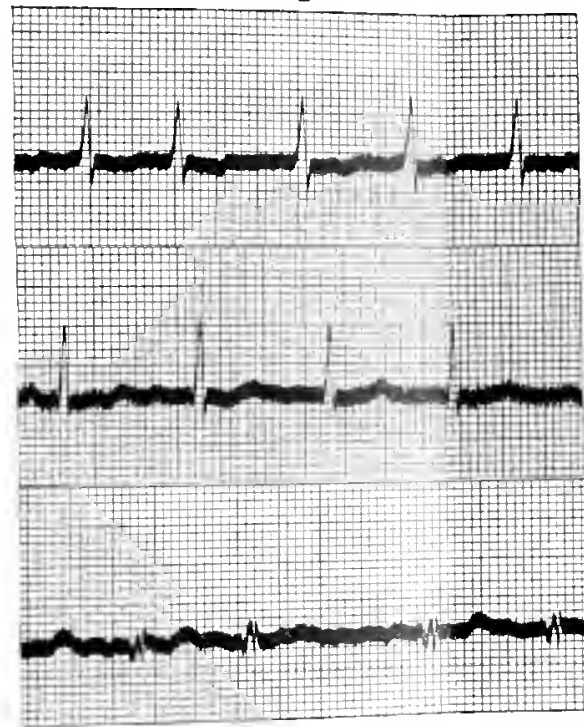
(Cable Fringe and Junction Induced by Digitalis on Human Electrocardiogram)

A



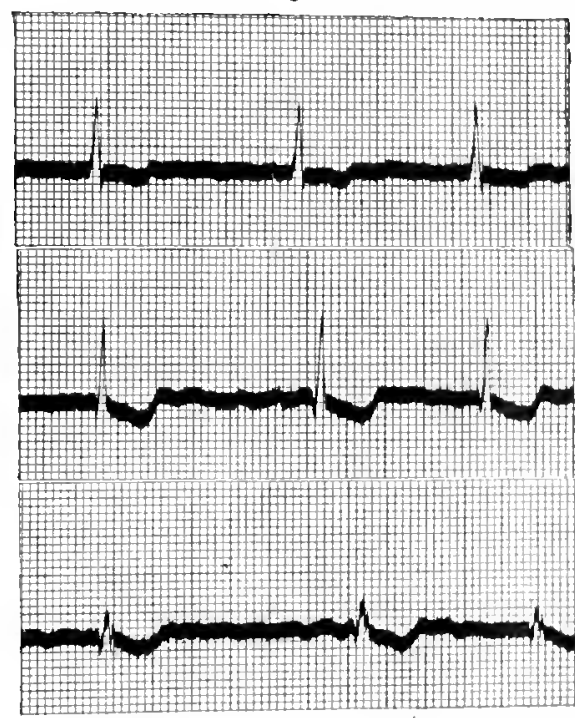
NOV1 DIGITALIS 3.2 gm.

B



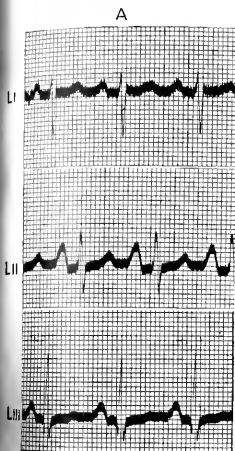
NOV.20 CONTROL

C

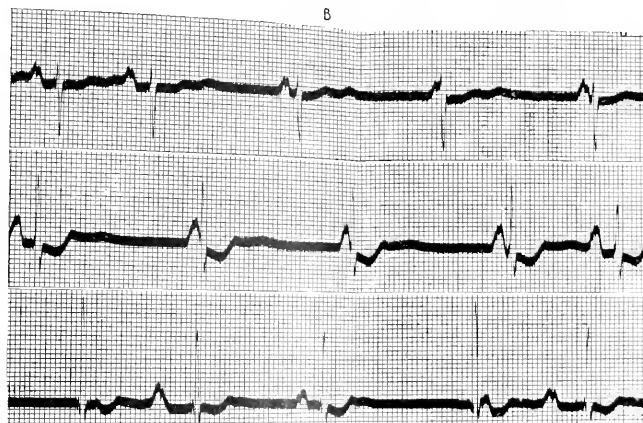


DEC.7 DIGITALIS 3.6 gm

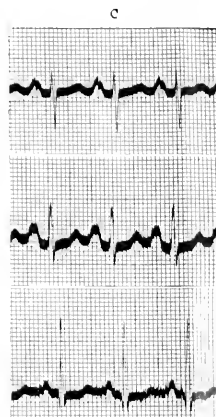
FIG. 5. (Cohn, Fraser, and Jamieson: Influence of Digitalis on Human Electrocardiogram.)



OCT. 19, 1914  
RATE 97.3  
P-R 0.22

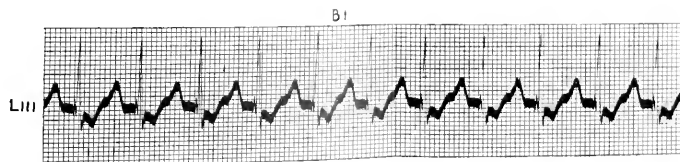


NOV. 6, 1914 DIGITALIS 2.5 gm.  
48



DEC. 29, 1914

0.2



ATROPINE 1.0 mgm  
RATE 120  
P-R 0.2

FIG. 6

(Cohn, Fraser, and Jamieson: Influence of Digitals on Human Electrocardiogram.)



# A STUDY OF THE EFFECT OF SENSITIZATION ON THE DEVELOPMENT OF THE LESIONS OF EXPERIMENTAL PNEUMONIA IN THE RABBIT.\*

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The analogies between the phenomenon of anaphylaxis in the laboratory experiment and the development and course of spontaneous pneumonia in man are, perhaps, more striking than those shown by any other infection. In pneumonia, moreover, conditions of hypersusceptibility might conceivably be one of the factors or the dominant factor, not only in the systemic febrile reaction but also in the localized and unique lung reaction such as is found only in man. The following study records a series of experiments in which an attempt was made to ascertain whether any definite and constant relationship between conditions of hypersusceptibility and the development of pneumococcus lesions of the lobar type in animals could be established.

Wadsworth,<sup>1</sup> in 1904,—before the phenomenon of anaphylaxis had been associated with infections,—stated that typical lobar lesions developed in a considerable number of partially immunized rabbits after tracheal injection of small quantities of pneumococcus culture. These lesions, absent when normal rabbits had been injected, were also much less extensive or lacking when highly immunized animals were used. Viewed from our present knowledge of anaphylaxis the acute lung reaction might possibly have been due to a state of hypersusceptibility unintentionally caused while attempting to induce a slight immunity as a predisposing condition to the development of the lobar type of lesion.

Typical lobar pneumonia has been successfully incited in the dog by Lamar and Meltzer<sup>2</sup> and others by intrabronchial insufflation of large quantities of pneumococcus culture. In rabbits the reaction has been induced experimentally with difficulty, as, owing to their extreme susceptibility to pneumococcus infection, a general bacteremia without characteristic lung involvement is usual.

\* Aided by a grant from The Rockefeller Institute for Medical Research. Received for publication, November 5, 1914.

<sup>1</sup> Wadsworth, A., *Am. Jour. Med. Sc.*, 1904, cxxvii, 851.

<sup>2</sup> Lamar, R. V., and Meltzer, S. J., *Proc. Soc. Exper. Biol. and Med.*, 1909-10, vii, 102; *Jour. Exper. Med.*, 1912, xv, 133.

Rasquin<sup>3</sup> stated that tracheal injection of pneumococci gave typical pneumonias in only 7 to 8 per cent. of his rabbits, but when serum from a dog immunized to rabbit serum was added to the culture, 96 per cent. developed typical catarrhal lesions. Winternitz and Hirschfelder,<sup>4</sup> however, by tracheal insufflation of large amounts—4 to 5 c.c.—of concentrated culture were able to obtain extensive fibrinous involvement in a large proportion of fairly small rabbits.

Friedberger<sup>5</sup> and later Schlecht and Schwenker<sup>6</sup> reported a cellular bronchopneumonia produced by spraying horse serum into the tracheas of sensitized guinea pigs. Anaphylactic shock and pneumonic changes in the lungs of sensitized guinea pigs following intratracheal injection of small amounts of horse serum also have been reported by Ishioka.<sup>7</sup>

While the rabbit has proved more resistant to sensitization than the guinea pig, and the results generally less constant and less marked, definite anaphylactic reactions with bacterial proteins have been obtained. Neufeld and Dold<sup>8</sup> and Rosenow<sup>9</sup> incited acute toxic symptoms in the rabbit similar to those of anaphylaxis by using a pneumococcus culture and normal or immune serum-complement mixture, such as Friedberger had obtained with other bacteria. The toxic split products of pneumococci obtained by Vaughan, the autolysates of Rosenow, and the lytic bile extracts of Cole have all caused acute toxic reactions in the rabbit. Apparently in none of the experiments with bacteria has the toxic dose been given tracheally, nor has the local reaction called forth by it been studied.

In the present study two hypotheses have been kept in mind: one which would be strengthened by negative results, that in lobar pneumonia, notwithstanding its extraordinarily rapid and exceptional course, the progress is still a definitely progressive one and not dependent on conditions such as give rise to the sudden and eruptive phenomena of anaphylaxis; the second, suggested by the abrupt onset and sudden termination of the disease, by certain local reactions, and by the not infrequent clinical history of previous infection suggestive of sensitization, that its unique and striking features are of an anaphylactic nature. In the latter case pneumonia may be considered either as mainly a bacteremic or toxemic condition, the lung lesions being a local and comparatively unimportant feature of the disease as a whole, and the anaphylactic reaction a general and systemic phenomenon. Or, the lungs with their system of tubes

<sup>3</sup> Rasquin, E., *Arch. de méd. expér. et d'anat. path.*, 1910, xxii, 804.

<sup>4</sup> Winternitz, M. C., and Hirschfelder, A. D., *Jour. Exper. Med.*, 1913, xvii, 657.

<sup>5</sup> Friedberger, E., *Deutsch. med. Wchnschr.*, 1911, xxxvii, 481.

<sup>6</sup> Schlecht, H., and Schwenker, G., *Deutsch. Arch. f. klin. Med.*, 1912, cviii, 405.

<sup>7</sup> Ishioka, S., *Deutsch. Arch. f. klin. Med.*, 1912, cvii, 500.

<sup>8</sup> Neufeld, F., and Dold, H., *Berl. klin. Wchnschr.*, 1911, xlviii, 55.

<sup>9</sup> Rosenow, E. C., *Jour. Infect. Dis.*, 1911, ix, 190.



and air spaces may provide a particularly favorable environment and may act as a coöperating test-tube in which the cell complex furnishes the antibody, and the invading pneumococci the toxic agent. The resulting toxic substances, acting locally, might then incite exudative reactions in the lung, and on being absorbed give rise to the characteristic febrile reaction, the bacteremia being a secondary and transitory phenomenon.

*Methods.*—The pneumococcus organisms used were A, a virulent strain (the Neufeld culture), obtained through the kindness of Dr. Cole of the Hospital of The Rockefeller Institute; B, one of moderate virulence, but which had given evidence of unusual toxicity; and AA, an avirulent culture of A, attenuated by long continued growth on agar. Derivatives of eighteen to twenty-four hour meat infusion broth cultures were used for sensitization. The culture was centrifugalized and the supernatant fluid passed through a Berkefeld and then a Pasteur filter. The sediment was washed twice, suspended in salt solution equal to one-half or one-quarter of the original culture, and heated for thirty minutes at  $52^{\circ}$  to  $55^{\circ}$  C. Usually 0.1, 0.5, 7.5, and 15 cubic centimeters of filtrate or dead cell suspension were given intravenously to groups of rabbits. Two weeks later one cubic centimeter of eighteen to twenty-four hour live broth cultures was given tracheally to the rabbits. The same or a larger volume of concentrated suspension of living pneumococci cultivated aerobically in broth, or anaerobically under oil, or in large Petri dishes, was later used in a second series of rabbits. The effect of repeated sensitizing or toxic doses was also tested.

In studying the reactions incited by tracheal injection of serum and culture, mixtures were made of one cubic centimeter of live broth culture, and 0.1 or 0.5 of a cubic centimeter of sera from normal rabbits, or from animals immunized with pneumococcus filtrates, or with dead cells. These mixtures were usually injected immediately, and after incubation at  $37^{\circ}$  C. for periods of one to four hours.

The rabbits were etherized lightly and at the time of the tracheal injection tilted so that the natural course of the fluid would be into the left lung. Temperatures were taken before tracheal injection and once or twice a day afterward. If not already dead, the ani-

mals, with few exceptions, were killed at the end of forty-eight hours. At autopsy smears were made from the heart's blood and cultures from the pleural cavity, heart, liver, spleen, and trachea. Paraffin sections were made of all lungs, which had been previously distended and preserved in 95 per cent. alcohol or 10 per cent. formalin.

In the first series of experiments preliminary intravenous injection of culture filtrates or dead cells for purposes of sensitization was followed after an interval of two weeks by tracheal injection of living organisms.

#### SENSITIZATION WITH CULTURE FILTRATES.

*Experiment 1.*—A series of 20 rabbits received intravenously 0.1, 0.5, 7.5, or 15 c.c. of culture filtrate of the attenuated culture AA. Two weeks later 1 c.c. of a living broth culture of the same avirulent organisms was injected tracheally. None developed symptoms of anaphylaxis and all were killed after 48 hours.

While there was considerable variation in the lung reaction, in no instance was the lobar type of lesion approached, nor was there any apparent connection between the amount of the preliminary inoculation and the extent and nature of the reaction.

*Experiment 2.*—Similar preliminary treatment of 8 rabbits with filtrates of the same avirulent organisms, but with the virulent culture substituted in the tracheal injection, resulted in even less lung involvement.

*Experiment 3.*—In a third series 6 rabbits received both sensitizing and tracheal injections of the moderately virulent, though highly toxic strain B. The febrile reaction was acute. Only one rabbit died within 48 hours, although the lung involvement was much more diffuse and extensive than in the other series. The reaction, however, was apparently independent of the amount of filtrate previously received, and the unsensitized controls developed similar though, on the whole, less marked reactions.

*Experiment 4.*—In striking contrast was the almost complete absence of diffuse exudative lesions in the series of 16 rabbits receiving filtrates of the virulent strain A followed by tracheal injection of live organisms of the same virulent strain. The animals given the larger sensitizing doses had acquired considerable immunity, as they were still alive after 48 hours, whereas those sensitized with similar doses of the avirulent strain in experiment 2 died.

#### SENSITIZATION WITH DEAD PNEUMOCOCCUS CELLS.

*Experiment 5.*—19 rabbits which had received 0.1, 0.5, 7.5, and 15 c.c. of a killed suspension of the avirulent organisms AA were injected tracheally with 1 c.c. of a live broth culture of the same avirulent culture.

While there was more congestion and somewhat more lung involvement than in the corresponding filtrate series, the reactions did not differ materially in the different groups.

*Experiment 6.*—Eight additional rabbits which had been treated with the same dead cell suspension of the avirulent culture, but injected tracheally with the virulent organisms of the same strain, failed to develop marked exudative lesions. Treatment with large doses of dead cells had apparently a stronger protective action than similar treatment with filtrates of the avirulent culture, as two rabbits in this series were alive after forty-eight hours.

*Experiment 7.*—The reaction incited by the moderately virulent strain B, when given in both sensitizing and tracheal injections, was studied in 12 rabbits. The majority developed extensive exudative lesions in which areas of hemorrhage and necrosis were quite numerous. In four unsensitized controls, similar, but on the whole less extensive, areas of consolidation were found. The lesions, while more marked, closely resembled those in the corresponding filtrate series.

*Experiment 8.*—In this experiment in which sensitization with dead cells of the virulent culture was followed by tracheal injection of the same strain in the virulent state, 4 out of 14 rabbits developed extensive exudative lesions, in which fibrin and polymorphonuclear leucocytes were abundant. These lesions, however, were not confined to rabbits which had received the same sensitizing doses. While the two untreated controls died in 24 hours without marked lesions, the protective action of the sensitizing injections was shown by the survival for at least 48 hours of a number of the treated rabbits which had received the larger doses.<sup>10</sup>

In the following series concentrated cultures and culture material were used under similar conditions of experiment.

*Experiment 9.*—Of 16 rabbits treated with the usual concentration of dead cells, 4 received intravenous, and 12 tracheal injections of a concentrated suspension of living virulent organisms grown on agar. None showed anaphylactic symptoms. The lung reaction when present resembled that usually associated with foreign body pneumonias.

*Experiment 10.*—17 rabbits which had already received sensitizing and toxic injections were reinjected tracheally with a concentrated suspension of pneumococci grown in broth anaerobically under oil. No extensive lesions developed.

*Experiment 11.*—8 rabbits received single or repeated sensitizing injections of dead cells followed by intravenous injections of a concentrated suspension grown on agar. In one rabbit which had received a single sensitizing injection of 5 c.c., the toxic dose of 2 c.c. was immediately followed by symptoms resembling marked anaphylactic shock followed by complete recovery in about 30 minutes. The lungs at autopsy, 48 hours later, showed practically no involvement. In another series of 7 animals, one rabbit sensitized with 5 c.c. of concentrated dead cells and reinjected with 3 c.c. of a concentrated suspension of live organisms grown on agar died in less than 5 minutes, with symptoms resem-

<sup>10</sup> Additional sensitized rabbits in this or other series died in the latter part of the interval before tracheal injection; a number died of an infection present at the time, but less fatal to normal rabbits, suggesting that the animals, while they had acquired more tolerance to the pneumococcus, were so injured that they had become more susceptible to other infections.

bling anaphylaxis. No unusual reaction was found in the lungs on microscopical examination.

*Experiment 12.*—For the purpose of comparison 12 rabbits were sensitized with repeated injections of horse serum and reinjected at intervals intravenously or tracheally with the same serum. Anaphylactic shock developed in most of the animals receiving the toxic injection intravenously. Of those injected tracheally one was prostrated immediately after the injection, but recovered in half an hour. Killed 48 hours after the last tracheal injection. The sensitized rabbits' lungs showed possibly more peribronchial reaction than the unsensitized controls which had received tracheal injection, but the difference was not striking and no diffuse lesions of the lobar type were found.

In the preceding experiments active sensitization with filtrates or dead cells of strain A in its highly virulent or avirulent state followed by tracheal injection of live virulent or attenuated cultures of the same race did not result in a definitely increased lung involvement. When a second strain B, of much less virulence, was used, marked exudative lesions, varying to some extent, developed in the rabbits previously sensitized with filtrates or dead cells of the same strain, but the untreated controls also developed similar though possibly less extensive reactions. Since these experiments with active sensitization failed to bring out any very definite relationship between sensitization and the character of the lung lesions, the effect of passive sensitization by means of tracheal injection of different sera and culture mixtures was next studied.

#### TRACHEAL INJECTION OF MIXTURES OF SERUM AND CULTURE.

*Experiment 13.*—Mixtures of 0.5 c.c. of fresh pooled normal rabbit sera and 1 c.c. of living attenuated culture, freshly mixed or incubated, were given tracheally to 8 rabbits. The animals almost at once developed marked signs of discomfort, such as restlessness, dyspnea, and prostration, their temperatures falling at least 3.2° F. The control rabbit, however, receiving 1 c.c. of culture diluted with 0.5 c.c. of sterile broth developed similar, though less marked, symptoms. All recovered in half an hour. The control and the rabbit receiving the mixture incubated for over 3 hours died in about 48 hours. The seven others died within 4 hours of each other in sudden and violent paroxysms, about 24 hours after the tracheal injection. Pneumococci were found in all cultures. A small rabbit injected intravenously with 1 c.c. of a transfer from the culture used in this experiment was unaffected.

The control's lungs showed some peribronchial infiltration. Two rabbits which had received serum mixtures developed extensive, two less marked diffuse fibrinous lesions. Repetition of the experiment failed to incite unusual symptoms, though in the lung of several rabbits small lesions of a fibrinous character were found.

*Experiment 14.*—Similarly mixtures of normal rabbit sera and virulent living culture were given tracheally to 21 rabbits. No immediate symptoms developed, but a number died in acute paroxysms 24 hours after tracheal injection. In the first series of these rabbits receiving a mixture containing 0.5 serum, which had stood at room temperature for about 2½ hours, one rabbit developed a typical fibrinous lobar pneumonia, the other smaller diffuse lesions. In later series the proportion of rabbits developing diffuse and fairly extensive lung involvement was, however, much smaller. Incubation of the mixtures for 2 to 3 hours appeared to favor a slightly increased lung reaction.

*Experiment 15.*—Of 12 rabbits receiving mixtures of avirulent living culture and sera from rabbits immunized with culture filtrates of the virulent strain, none showed immediate symptoms, but 7 died within 24 hours of injection—the majority in violent convulsions. The control which had received culture without serum died while not under observation in less than 48 hours. While four serum mixture rabbits developed considerable diffuse fibrinous involvement, the remainder, including the control, showed little or no exudative reaction.

*Experiment 16.*—The effect of virulent cultures and sera from rabbits immunized with filtrates of the same virulent strain A was tested on 26 rabbits. Of four receiving mixtures which had stood at room temperature 2 to 3 hours and in which the sera was three weeks old, two developed typical fibrinous lobar involvement. In the other series one rabbit showed extensive, and quite a number smaller diffuse fibrinous lesions. While the animals receiving the larger amount of sera possibly showed more lung reaction, the period of incubation apparently exerted little or no effect in this experiment.

*Experiment 17.*—The experiment was repeated, 11 rabbits receiving attenuated cultures and sera from animals immunized to dead pneumococcus cells. As before, the mixtures were incubated for varying periods. All the rabbits were still alive at the end of 48 hours. The lung reaction was distinctly less than in the corresponding normal or filtrate sera and avirulent culture experiments.

*Experiment 18.*—A series of 22 rabbits was given similar mixtures of virulent culture and sera from rabbits immunized to dead cells. The sera would seem to have exerted a certain amount of protective action, as over half of the rabbits were alive at the end of 48 hours. None developed extensive lesions.

The effect of intravenous inoculation of normal and immune sera immediately before tracheal injection of live culture was tested in the following experiment.

*Experiment 19.*—11 rabbits were inoculated intravenously with 0.1 or 0.5 c.c. of normal rabbit or horse sera, or with sera from rabbits immunized with filtrates or with dead cells. This was immediately followed by tracheal injection of living virulent organisms. Of the three rabbits, which had received injections of sera from rabbits immunized with filtrates, all died practically 20 hours after injection in acute paroxysms. One of three rabbits, receiving sera from rabbits immunized with dead cells, was alive after 48 hours; the others died while not under observation. None developed diffuse lesions.

The distribution in the lungs of material injected through the trachea, and the resulting injury, must necessarily vary considerably. While these mechanical factors were undoubtedly responsible for certain differences in the bronchopneumonic reaction, they did not obscure the results. Tracheal injections of only 1 or 1.5 cubic centimeters had been given to avoid reactions caused by excessive quantities of concentrated culture such as Winternitz and Hirschfelder had obtained in normal rabbits, and also because it seemed desirable to approach more closely conditions of spontaneous pneumonia in man. Similarly, small sensitizing and non-concentrated toxic doses had been carefully tested, as it was thought a hypersensitive condition favorable to the development of a definite cellular reaction in the lung might be present, although acute or even mild anaphylactic symptoms were entirely lacking.

Active sensitization by previous intravenous injection of pneumococcus filtrates or dead cells failed either to hasten death or to increase markedly the lung reaction, although beginning with as small sensitizing doses as 0.1 of a cubic centimeter the amount injected had been increased until there had been produced an immunity sufficient to protect. In fact, the controls which had not received preliminary treatment were usually the first or among the first to die, indicating that the chief effect of the preliminary treatment was to increase materially the resistance of the animals, especially when large doses were used. While filtrates of the non-virulent culture AA, although originally derived from the extremely virulent strain A, failed, even in large doses, to protect from tracheal injection of virulent organisms of the same strain, and while the protective action of the moderately virulent strain B could not be gauged, as both controls and treated rabbits, with one or two exceptions, live for forty-eight hours, in no instance was death hastened as a result of sensitization.

Comparison of the histological changes in the lungs of treated rabbits and untreated controls also failed to show striking or constant differences, though it appeared that preliminary treatment, especially with dead cells, possibly favored a somewhat more diffuse reaction. In the two experiments in which extensive lesions were found, not only in the treated animals but also to a lesser extent

in the untreated controls, the strain used, though of moderate virulence, was considered especially toxic as compared with the more virulent strains studied. Extremely virulent organisms, on the other hand, apparently encountered little resistance from the lung tissues, but passed immediately into the circulation without inciting exudative reactions. In certain instances where large preliminary doses had been given, the lung involvement was also proportionately greater.

These results would seem to bear out in some measure the view that in pneumonia the lung reaction is partly dependent on a favorable equilibrium obtaining between the natural or acquired resistance of the host and the degree of virulence and toxicity of the organisms rather than on an anaphylactic state.

The reactions, both systemic and local, incited by tracheal injection of serum culture mixtures were somewhat more marked. A supposedly attenuated culture to which normal sera had been added caused immediate but transient symptoms suggesting mild anaphylactic shock. Seven of the eight rabbits, however, died in violent convulsions twenty-four hours later. These acute paroxysms, which closely resembled those of fatal anaphylaxis, also occurred in other series about twenty to twenty-four hours after serum mixtures had been injected.<sup>11</sup> The animals suddenly showed extreme restlessness, threw themselves about their cages, making violent running or jumping motions and often crying out. Death occurred within a few minutes of the onset of the symptoms, the heart continuing to beat for some time after respiration had ceased. Examination of the lungs failed, however, to show any definite connection between these paroxysms and the development of exudative reactions.

It is difficult to account for the sudden death of such a large number of the animals at practically the same time, although the interval after inoculation varied in the different experiments, except as the result of some phase of delayed anaphylactic shock. This delayed reaction might be due to several factors, but not to the tracheal method of injection alone, because similar reactions also

<sup>11</sup> In other experiments similar results were noted as early as 10 to 12 and as late as 32 to 34 hours after inoculation.

occurred after intravenous inoculation. The capsule of the pneumococcus cell which is protective according to Welch, and the interval required for the production of sufficient toxin, whether from the body fluids or from the pneumococcus cells through growth, might be important factors.

The local tissue reactions incited by the serum culture mixtures, particularly those containing normal serum or serum from rabbits immunized to filtrates, were also somewhat more marked and developed in a larger proportion of animals than in the experiments with active sensitization. Several rabbits developed typical fibrinous lobar pneumonia, and a number of others less extensive but diffuse exudative lesions. These were partly offset, however, by the partial or almost complete absence of exudative reactions in the other rabbits of the experiments, which had received similar treatment.<sup>12</sup>

Throughout the experiments the absence of uniformity in the lung reaction was especially striking. Controls in a few instances developed more involvement than certain of the treated rabbits of the same experiment, while in several series the test animals ran the whole gamut of reaction without apparent relation to the special character of preliminary treatment or tracheal injection.

The extreme sensitiveness of the pneumococcus to any alteration in its environment was repeatedly noticed. While the causes of fluctuation in virulence and toxicity are obscure, they not infrequently seemed closely associated with growth activity. That extremely subtle reactions occur within the body seems probable, and until the laboratory methods more nearly duplicate conditions present in the body, where the cellular elements and body fluids undoubtedly play an important part, similar and constant results can hardly be expected. In the present experiments when body conditions were more nearly approached the lung reaction was generally increased. It is of interest that in Wadsworth's experiment with partial immunization, the pneumococci used in the tracheal injection were grown in equal parts of normal rabbit serum and broth, while Winternitz and Hirschfelder used a 5 per cent. serum medium.

<sup>12</sup> For purposes of comparison tests with heterogeneous sera were made. Three rabbits received tracheally mixtures of culture and sera from a horse immunized to live cultures. Two developed diffuse and fairly extensive exudative lesions, one a very slight reaction.



Although an anaphylactic or hypersensitive condition in the host may possibly enable the invading pneumococcus to gain its first foothold, similar conditions might equally well underlie the development of a streptococcus infection, but would not necessarily determine in either case the type of lesion that later develops. In this study the essentially progressive character of the lung lesion was frequently brought out, all degrees of exudative reaction being found from small peribronchial foci merging into patchy or confluent lesions to the typically lobar involvement.

#### SUMMARY.

While preliminary treatment with culture filtrates or dead cells of the extremely virulent strain A gave rise to varying degrees of immunity, the exudative lung lesions developing after tracheal injection with live organisms of the same virulent strain were not strikingly increased in any group or series of these rabbits. Despite carefully graduated dosage in the preliminary treatment, none of the animals developed symptoms of a definitely anaphylactic nature.

In similar experiments following sensitization with the attenuated avirulent culture AA, tracheal injection of virulent or avirulent organisms of the same strain failed to incite any definite increase in the exudative lung reaction. In none of the rabbits were symptoms resembling anaphylaxis noted. The immunity which was induced by the larger sensitizing doses of culture filtrates of the strain in the virulent state was lacking when similar doses of the culture filtrates of organisms in the non-virulent state were used.

Extensive lesions developed in both sensitized and unsensitized rabbits when the strain used in the tracheal injection was one apparently combining moderate virulence with exceptional toxicity, indicating that the exudative lung reaction was one of adjustment rather than of acquired hypersusceptibility.

When, in the experiments, small amounts of sera from normal rabbits, or from animals immunized to culture filtrates, were added to the culture before tracheal injection, an increased fibrinous lung reaction was frequently found.

The present study would seem to give some ground for the view that while in pneumonia a hypersensitive condition probably takes

some part in the inception of the infection, the subsequent development of the diffuse exudative reaction in the lung is not directly due to an acquired hypersusceptibility, but to intrinsic qualities possessed by the pneumococcus itself.

This study was carried on in the Department of Bacteriology of the College of Physicians and Surgeons of Columbia University, New York, and later, through the courtesy of Dr. Charles Norris, in the Bellevue Hospital Laboratories. It was completed in the Laboratories of the New York State Department of Health at Albany. I am greatly indebted to Dr. Augustus Wadsworth for his advice and criticism.

# THE VISUAL CORTEX, ITS LOCALIZATION, HISTOLOGICAL STRUCTURE, AND PHYSIOLOGICAL FUNCTION.\*

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The cytoarchitectonic study of the cortex has reached a high development during the last twenty to thirty years. In earlier times it was believed that the whole cortex represented more or less a homogeneous organ, but it has since been concluded that this is not the case. Though the same architectonic ground type may be found in the entire cortex, yet in different regions certain histologically well characterized modifications of the cortex exist, with small variations, not only in man but in all higher mammals. In the most characteristic and well marked type of cortex belongs that portion called by Elliot Smith the area striata. On account of its special morphological character and its physiological importance, that is, its relation to the act of seeing, the area striata has been investigated with special attention. In this paper I shall discuss its localization, anatomical structure, and physiological function.

It was known to the earlier investigators, Gennari, Vicq-d' Azyr, and Bailarger, that the cortex of the fissure calcarina had a different structure from the rest of the cortex. One can readily see with the naked eye in the gray substance a stripe of white fibrous matter lying tangentially. This is called the stripe of Gennari or of Vicq-d' Azyr. The characteristic marks of the morphology and order in this part of the cortex were recognized much later by Meynert. A number of authors (Betz, Leonowa, Hammarberg (1), and Schlapp) have partly abandoned the division of layers after Meynert, but they have given no new point of view. They have also distinguished eight layers in the cortex. Cajal, who has studied the cortex with the help of the chrome-silver method of staining, has distinguished nine layers in the division of the visual cortex, based principally on the fine morphology of the cells.

Brodmann (2) has given the most detailed study of the cortex in general and especially of the calcarine type. He has not only studied each type histologically, but has defined its extension topographically on the surface of the

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cortex and has produced what may be called brain maps. The two most characteristic types of the cortex are the giant pyramid types, called the area gigantopyramidalis, and the calcarine type, named area striata. He has studied them in their variations and extensions in man and primates, and also in most classes of mammals. I shall keep chiefly to his descriptions in what follows, so that the connection of each layer in the area striata, to the sixth layer ground type, appears distinctly as six fundamental primary types of the cortex. It seems to me that Brodmann is clearer on this point when he divides the area striata into six layers, of which the fourth is subdivided into three secondary layers.

*I. Lamina Zonalis, or Moleculosis.*—This is well marked; it contains single small nerve cells of different shape, partly round, partly star-shaped, with short points. It does not differ essentially in its construction from the first layer of the rest of the cortex.

*II. Lamina Granularis.*—The external granular layer, or layer of small pyramids. This is sharply outlined in the cortex of adults, especially at its junction with the lamina zonalis. It consists of a narrow layer of granular or small pyramidal cells lying closely together. The transition from the small to the medium sized pyramids of the third layer goes on gradually, so that often it is difficult to distinguish here two different layers as in the rest of the cortex; that is why it is often included with the third layer.

*III. Lamina Pyramidalis.*—The layer of the medium sized pyramids is also small and poorly developed. The pyramids do not reach, anywhere, the size of the neighboring area occipitalis. Already in the inner part of this layer appear small round nerve cells called granules, whose number increases continually; at the inside edge of the pyramid layer they undergo a condensation which Brodmann considers as a special layer.

*IV. The Lamina Granularis Interna, Superficialis IVa.*—According to Brodmann, the undivided inner granular layer of the neighboring type, is divided in the visual cortex into a superficial and a deep sublayer; between the two a layer, poor in cells, is enclosed, the lamina granularis intermedia IVb. This latter is especially characteristic of the visual cortex.

In the Weigert preparation is here seen the stria of Vicq-d' Azyr or of Gennari, a tangential white stripe visible with the naked eye.

After being stained by Nissl's method this layer is seen as a light stripe, poor in cells. Here are the characteristic, small, medium sized, and star-shaped single cells.

Next comes the principal granular layer IVc, the lamina granularis interna of Brodmann.

In comparison with the preceding layer this one appears well marked, and about as broad as IVa and IVb together. It consists of numerous granules lying close to each other; there are also single elements of the neighboring layer called "guests"; star-shaped, spindle-shaped, and pyramid-shaped cells.

*V. The Ganglion Layer (Brodmann), or the Intermediary Layer (von Monakow).*—This forms again a clear layer of single, scattered cells, which contain separate large pyramidal cells, named Meynert's solitary cells. In fiber preparations there is seen also a small stripe of transverse fibers, the under stria of Vicq-d' Azyr or Gennari.

*VI. The Multiform Layer.*—This layer, the layer of polymorphic elements of von Monakow, represents a clear and characteristic part, which is marked towards the outside by the lamina ganglionaris and towards the inside by the stripe of white matter or fibers. In spite of its narrowness it consists of two layers; an outside layer of compact, mostly three cornered cells, the lamina triangularis, and an inner layer of loose spindle-shaped cells, the lamina fusiformis.

It is therefore characteristic of the area striata of man that the large pyramidal cells in the pyramidal layer are missing. In the layer of star-shaped cells, the Gennari stripe and the large inner granular layer, also a well marked multiform layer rich in cells, and the closeness and smallness of the cells are characteristic of this type (text-figure 1). Brodmann studied the tectonic variations and the extension of the calcarine type of the cortex, not only in man but in all mammals. He worked out the homology of the area striata in all classes of mammals as far as they were examined. The area striata of the monkey is similar in structure to that of man. It is here also marked by a light layer of numerous star-shaped cells, and a broad inner granular layer in which star-shaped cells are scattered about.

In some monkeys, like *Cebus capucinus*, there is a different cytoarchitectonic structure which surpasses that in man. Here, inside of the star-shaped layer, is another layer of larger cells running into each other, so that this layer can again be divided into three secondary layers: a superficial layer, a dark middle layer, and a deeper light one.



I. Lamina zonalis.

II. Lamina granularis externa.

III. Lamina pyramidalis.

IVa. Lamina granularis interna superficialis.

IVb. Lamina granularis intermedia; here lies the stria of Gennari.

IVc. Lamina granularis interna profunda, or principal granular layer.

V. Lamina ganglionaris.

VIa. Lamina triangularis.

} VI. Lamina multiformis.

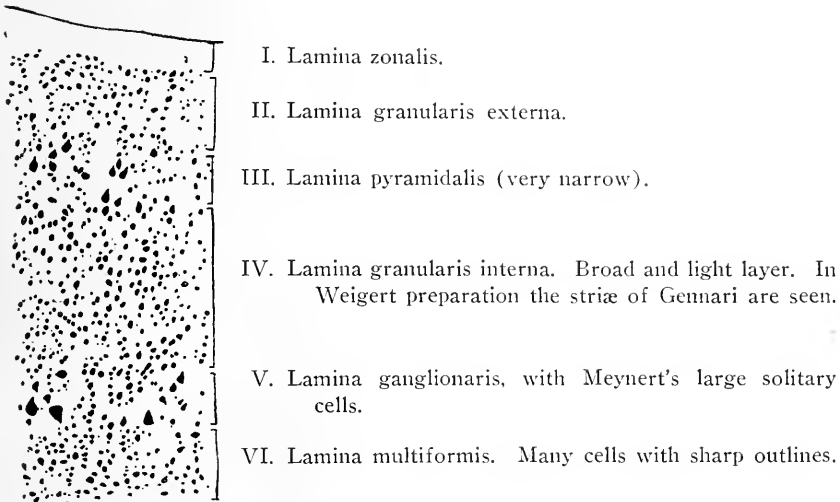
VIb. Lamina fusiformis.

TEXT-FIG. I.<sup>1</sup> Calcarine cortex. Man.  $\times 44$ .

<sup>1</sup> A list of the meanings of the abbreviations used in the text-figures is given at the end of the paper.

Besides the more advanced tectonic development of the calcarine cortex of some primates is the large number of mammals with a more or less undeveloped or retrogressed type (Brodmann). According to Brodmann, the insectivora are a highly undeveloped type.

In carnivorous animals (cat and dog) a special layer of star-shaped cells can not be distinguished (text-figure 2). The area stri-



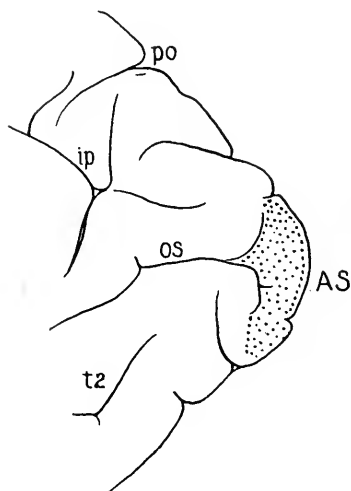
TEXT-FIG. 2. Calcarine cortex. Cat.  $\times 40$ .

ata is here especially characterized by a broad and clear inner granular layer in which star-shaped cells are scattered about. We find, on the whole, that the other characteristics are the same as those of primates; that is, a narrow, light sixth layer (lamina ganglionaris) with some large pyramidal cells. In general the cortex is very narrow; it is more compact, and the size of the cells is smaller. The transition of the area striata to the surroundings is not so well marked as in man and primates, but a gradual transition takes place. The pyramidal cells of the third layer become larger and go more deeply into the cortex; the inner layer becomes more compact and smaller. The lamina ganglionaris and multiformis are less frequently seen, or, briefly speaking, the ordinary six layer type appears again.

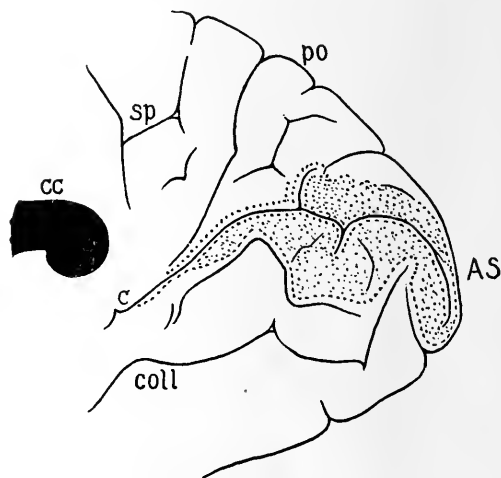
Until recently little was known about the exact localization of this type of cortex in man, and still less in animals. Bolton has de-

scribed (1900) the extension of the visuosensory area in the human cortex. His views were, in general, confirmed by Brodmann, Campbell, and others. It is now possible to give an exact division of this human cytoarchitectonic cortex field, as its boundaries are well marked in all directions.

Text-figures 3 and 4 show the extension of the area striata in the human occipital lobe (after Brodmann). Near the convexity it



TEXT-FIG. 3.

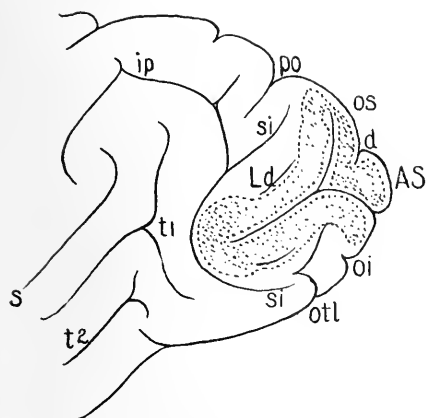


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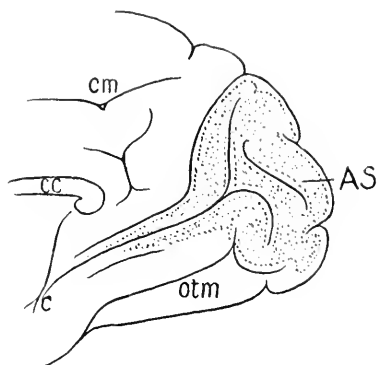
TEXT-FIGS. 3 and 4. Extension of the area striata in the human occipital lobe, convex and mesial surfaces; after Brodmann.

occupies only the extreme caudal end. On the mesial surface of the hemisphere it occupies the two lips of the calcarina, also the fissure retrocalcarina, which is hidden inside of the occipital lobe. It reaches from here to the neighboring regions; dorsally to the cuneus, and ventrally to the lobus lingualis. Anteriorly it diminishes quickly and ends after joining the fissure calcarina and the fissure paroccipitalis in the posterior part of the trunks of the fissure calcarina. The transition of the area striata into the structural type of the surroundings takes place suddenly; dorsally into the cortex of the cuneus, ventrally into that of the lobus lingualis, and on the convexity into the area occipitalis. The layer of star-shaped cells disappears, the



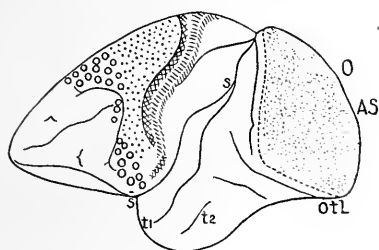


TEXT-FIG. 5.

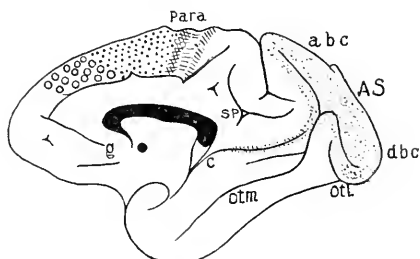


TEXT-FIG. 6.

TEXT-FIGS. 5 and 6. Extension of the area striata in the orang-utang, convex and mesial surfaces; after Brodmann.

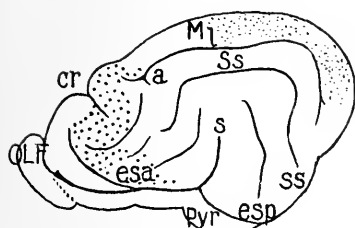


TEXT-FIG. 7.

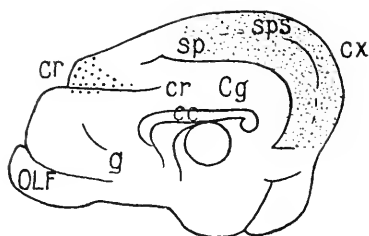


TEXT-FIG. 8.

TEXT-FIGS. 7 and 8. Extension of the area striata in the *cercopithecus*, convex and mesial surfaces; after Brodmann.



TEXT-FIG. 9.



TEXT-FIG. 10.

TEXT-FIGS. 9 and 10. Extension of the area striata in the occipital lobe of the cat, convex and mesial surfaces.

lamina granularis interna becomes narrower, the layer of pyramids becomes larger, and at the same time larger pyramidal cells appear in it.

The extension of the area striata shows, especially in the caudal and frontal ends, significant individual variations, which are confined to the mesial surface of the hemisphere. In the greatest number of cases it goes over on the convexity of the occipital lobe where it occupies the caudal pole.

In the anthropomorphous ape (text-figures 5 and 6 (orang-utang)) the area striata is extended over the mesial and convex surfaces of the occipital lobe approximately in equal parts.

In the *cercopithecus*, the long tailed monkey, the area striata occupies on the convexity the whole operculum between the sulcus similis and the fissure occipito temporalis lateralis. On the mesial surface the caudal pole is found around the ascending and descending branch of the fissure calcarina, while at the trunk of the fissure it soon disappears into the deep part of the fissure.

In carnivorous animals (text-figures 7 and 8) (cats and dogs) the area striata occupies principally the mesial and cerebellar surface of the occipital lobes. Their extension has been studied by Campbell, Brodmann, and Minkowski, whose results were the same (3, 4). The position of the area striata of the cat is marked by the caudal two-thirds of the gyrus marginalis in its mesial and lateral parts (text-figures 9 and 10).

The dog has a similar extension to that of the cat. The relation of this type of cortex to the act of vision was known by the earliest authors who had noticed after lesions in the neighborhood of the fissure calcarina lasting defects or scotoma in the field of vision.

Henchen and a number of other investigators have, by means of pathological lesions in the occipital lobes of man, marked out, as a clinical anatomical visual sphere, a zone whose destruction, in the minimum, is necessary to bring forth a maximum disturbance of vision. This zone consists in the cortex of the fissure calcarina, the neighboring lobes of the cuneus, and lobus lingualis; and therefore coincides with the extension of the area striata. The agreement about clinical and anatomical results that have been obtained has not remained without contradiction, as von Monakow explains

that he cannot accept, without further comment, Henehen's localization of the visual sphere in a relatively narrow part of the mesial surface of the occipital lobe, because of general pathological and anatomical reasons. He thinks it more likely that in all cases that have been described till now of the so called cortical lesion in the sphere of the calcarina, the optic radiation would also be damaged, that an extension which remains confined on the calcarine cortex is impossible, and therefore in calcarina lesions the occipital field cortex, which is separated from the primary occipital center, has really a larger extension and corresponds with the diseased area of the regio calcarina. von Monakow includes also in the anatomical clinical visual sphere, besides the regio calcarina with its specific cortex type, the first to the third occipital convolutions, the whole cuneus, the lobus lingualis, and the gyrus descendens. Experimental physiology seems to confirm this opinion. Minkowski's experimental research in the dog indicates that the clinical, anatomical visual sphere is covered with the cytoarchitectonic sphere, as lasting visual trouble can be caused only by extirpation of the area striata. Its total extirpation probably produces a maximal visual disturbance that can be reached from the cortex, while extirpation on the convexity of the occipital lobes, according to Minkowski, has as a consequence no, or only a short or quickly passing restriction of the visual field if they remain confined to the cortex and do not affect the optic radiation.

A further question arises: Is the cytoarchitectonic visual sphere close to the pathological anatomical sphere? von Monakow means by this the ideal convolution district in the occipital lobes whose whole destruction is just sufficient to bring forth a complete secondary degeneration of the primary optical centers and specially of the external geniculate body.

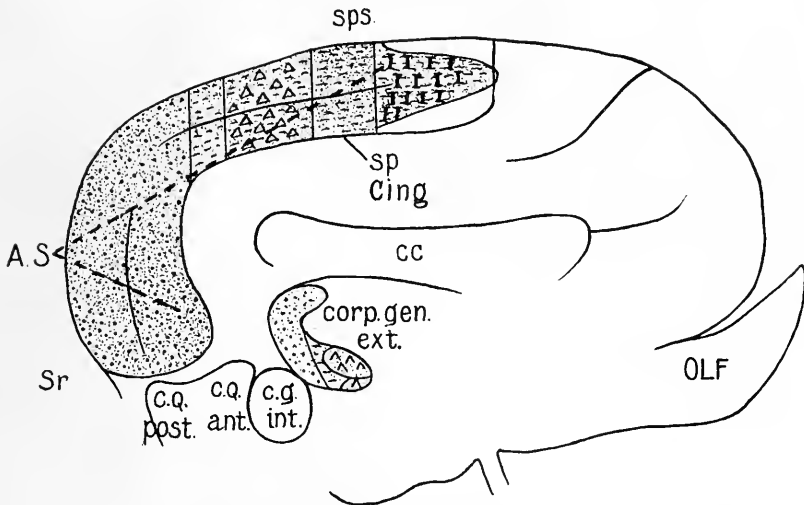
Here also human pathology can not give a precise answer. It is known, since the classical works of von Monakow, that the sub-cortical principal visual center of mammals, the corpus geniculatum externum, depends on the cortex, as it undergoes secondary degeneration through destruction of the occipital lobes. Until recently it was not possible to mark exactly on the cortex the district of representation of the corpus geniculatum externum. Only lately

has Minkowski succeeded in clearing up this point. He has experimental proof that the district of representation on the cortex of the cat by the corpus geniculatum externum is covered by the area striata; as only from this district can a secondary degeneration of the corpus geniculatum externum be produced by total extirpation of the area striata, the part most easily reached from the cortex, while the extirpation from the convexity of the occipital lobes outside the area striata produces in the corpus geniculatum externum a local degeneration, not a general volume reduction. If we consider the pathological anatomical visual sphere as the representative district of the corpus geniculatum externum, we may say that it is covered, in cats and dogs and probably in man, with the clinical anatomical and cytoarchitectonic visual sphere; that is, with the area striata.

A few words more about the projection of the corpus geniculatum externum and the area striata; that is, about the closer relation between certain parts of the area striata and the corpus geniculatum externum. It is known from human pathology that such a projection may exist; because through the destruction of the dorsal part of the visual cortex, that is, of the dorsal lip of the fissure calcarina and of the cuneus, the dorsal part of visual radiation, and, further, the dorsal part of the lateral fibers of the corpus geniculatum externum, the frontal mesial part degenerates secondarily; on the other hand, a secondary degeneration of the ventral part of the optic radiation and the caudal lateral part of the corpus geniculatum externum takes place after a lesion of the ventral half of the occipital lobes; that is, of the ventral lip of the calcarina and the lobus lingualis.

Minkowski has succeeded in showing the exact projection of the corpus geniculatum externum over the cortex and specially over the area striata of the cat. He has demonstrated that the frontal and upper parts of the area striata are in connection with the frontal part of the corpus geniculatum externum and the back, and at the same time the under parts of the area striata, with the back parts of the corpus geniculatum externum, so that after a partial extirpation of the area striata only the corresponding part of the corpus geniculatum externum degenerates secondarily. This projection is so sharply marked that it can be proved by a small cortex extirpation of 0.5 of a centimeter in diameter out of the area striata; because

after such an extirpation, a distinctly well marked island-shaped district of the corpus geniculatum externum undergoes secondary degeneration (text-figure 11).



TEXT-FIG. 11. Scheme of projection of the corpus geniculatum externum over the area striata in the cat.

#### SUMMARY.

In the study of the human cortex it seems well to follow the same primary divisions for the entire cortex, while in certain areas some layers may assume certain modifications; *viz.*, in the area striata it seems clearer to retain the sixth layer ground type, of which the fourth is subdivided into three secondary layers. The visual cortex, on account of its relation to the act of seeing, is an especially interesting field for study. About the calcarine fissure one can readily see with the naked eye a stripe of white fibers lying in the gray substance, called stria of Gennari or of Vicq-d' Azyr. This layer is considered by Brodmann in the second subdivision of the fourth primary layer.

The two most characteristic types of the cortex are the giant pyramid type, called area gigantopyramidalis, and the calcarine type, called area striata. The higher types of mammals afford excellent material for comparison.

Minkowski's experimental study of the dog indicates that the

clinical, anatomical visual sphere is covered with the cytoarchitectonic sphere; for lasting visual trouble can be caused only by extirpation of the area striata.

The subcortical visual center in mammals undergoes secondary degeneration through destruction of the area striata, and therefore depends on the cortex. Also certain parts of the corpus geniculatum externum have an exact projection over certain areas of the cortex.

#### ABBREVIATIONS USED IN THE TEXT-FIGURES.

cc,	corpus callosum.	abc,	ascending calcarina.
c,	calcarina.	dbc,	descending calcarina.
po,	parieto occipital.	g,	sulcus genualis.
ip,	intraparietal.	M,	gyrus marginalis.
os,	opercularis superior.	Ss,	gyrus supra Sylvius.
t <sub>1</sub> ,	first temporal.	Pyr,	lobus pyramidalis.
t <sub>2</sub> ,	second temporal.	Cg,	cingulum.
AS,	area striata.	Olf,	olfactory.
coll,	collateral.	l,	sulcus lateralis.
sp,	sulcus splenialis.	ss,	sulcus supra Sylvius.
s,	sulcus Sylvius.	esp,	sulcus ecto Sylvius posterior.
oi,	opercularis inferior.	esa,	sulcus ecto Sylvius anterior.
d,	descendens.	a,	sulcus ansatus.
si,	sulcus simialis.	cr,	sulcus cruciatus.
otl,	sulcus occipito temporalis.	sps,	sulcus supra splenialis.
cm,	calloso marginalis.	sr,	sulcus recurrens.
otm,	occipito temporalis medianus.	cx,	calcarine cortex.
otl,	occipito temporalis lateralis.	o,	operculum.
corp. gen. ext.,	external geniculate body.	Ld,	descendens lateralis.
c. g. int.,	internal geniculate body.	Para,	regio paracentralis.
c. q. ant.,	anterior corpora quadrigemina.	Cing,	cingulum.
c. q. post.,	posterior corpora quadrigemina.		

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# A STUDY OF THE PATHOGENIC PROPERTIES OF BACILLUS PROTEUS.\*

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PLATES 65 TO 67.

It is probable that no microörganism is encountered more frequently by those engaged in routine bacteriological work than some member of the *proteus* family, and yet there are few that at present attract less attention than these common bacteria. In fact, our standard text-books on bacteriology and pathology devote little or no space to these organisms.

The term *proteus* means a variety of form. Undoubtedly, many organisms have been classified as *proteus* bacilli which really do not belong to this class. Today the term *proteus* embraces such a large group of bacteria as to have no well defined limits.

## GENERAL CHARACTERS OF PROTEUS BACILLI.

Hauser (1) described three types of *proteus* bacilli, classified according to their action upon gelatin. *Bacillus proteus vulgaris* is a rapid liquefier, liquefaction appearing in cultures from six to eight hours old. *Proteus mirabilis* liquefies more slowly. Both of these types, according to Hauser, form zoöglea in gelatin cultures. *Proteus zenkeri* neither liquefies gelatin nor forms zoöglea in this medium. These organisms are aerobic bacteria, growing readily on all culture media.

It is not the purpose of the present study to attempt to clarify the somewhat confused classification of the *proteus* group of organisms. We shall limit ourselves to a brief description of those with which we have experimented.

The *proteus* bacilli are Gram-negative, actively motile organisms, whose morphological characters resemble those of the colon typhoid

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group. The Gram-positive, spore-forming organisms described by some writers, we believe, should not be classified with the *proteus* family.

The strains with which we have experimented showed beginning liquefaction within twenty-four hours. Liquefaction has never been observed earlier than twelve hours. Milk was curdled readily, and the coagulum redissolved in about ninety-six hours. Loeffler's blood serum was usually liquefied. When cultivated on the various sugar media, the *proteus* resembles the paratyphoid bacilli. Dextrose and maltose are fermented with acid and gas formation. Saccharose is unaffected by most strains. Lactose, as a rule, is not fermented.

Jordan (2) found that the ratio of hydrogen to carbon dioxide formed in dextrose media is characteristic of the *proteus*. According to this author the amount of hydrogen formed always exceeds that of carbon dioxide; and this fact may be used as an aid in identification. Indol is formed in very perceptible proportions.

Widely divergent opinions prevail relative to the pathogenic properties of the *proteus* organisms. Many authors regard them as obligatory saprophytes. There is ample evidence in the literature, however, to show that they may at times become pathogenic.

In reviewing the literature in an attempt to determine precisely with what diseased conditions the *proteus* has been observed, one is handicapped by the imperfect descriptions of the organisms termed *proteus*. In fact, in some cases only the morphology of the germ is discussed, and it appears that a few of the earlier writers have felt justified in classing any pleomorphic bacillus as a *proteus*.

Foà and Bonome (3) recovered an organism which they believed to be the *proteus* from a case of volvulus in the year 1889. About a year later Krogius (4) reported a case of cystitis caused by the *proteus*. In the same year Schnitzler (5) reported a series of nine cases of cystitis due to the same organism. Jaeger (6) studied a series of ten cases of Weil's disease, some of which came to autopsy, in infections which occurred in the garrison at Ulm. He concluded that Weil's disease was a specific infectious disease due to *Bacillus proteus*.

In this country a *proteus* infection was described as early as 1892 by Flexner (7), who isolated *proteus vulgaris* from a case of peritonitis which had terminated fatally. Since then it has been found in a multitude of diseased conditions, such as pyelonephritis, prostatitis, enteritis, abscesses of various localities, caries of the bone, pleuritis, meningitis, arthritis, osteomyelitis, and gangrene. Not only man but some of the domestic and even the cold-blooded animals are subject to *proteus* infections. Jensen (8) has repeatedly found the *proteus* to



be the cause of diarrhea of calves. Jaeger described an epidemic of fowls in which the *proteus* likewise was found to be the infectious agent. He isolated this organism from chickens, geese, and ducks which had succumbed to the infection. In the year 1897 Wyss (9) studied an epidemic among the fish in Lake Zurich, which was, he believed, caused by *proteus vulgaris*. He recovered the organism from thirteen carcasses.

#### SOURCES OF PATHOGENIC PROTEUS STRAINS.

The first *proteus* strain with which we experimented was isolated from a laparotomy wound in a case which subsequently terminated fatally. The *proteus* was repeatedly isolated from this patient in pure culture. The second strain was isolated from a severe eye infection following a cataract operation. A third strain was isolated from an infection of the finger, believed to have been contracted at an autopsy. A fourth was isolated from the heart's blood of a case which had died from peritonitis following a gunshot wound of the intestines. The fifth strain was from a case of gangrene of the lung.

These organisms all proved to be pathogenic for rabbits. In our preliminary report (10) we have called attention briefly to some of the histological changes produced by *Bacillus proteus*.

Below we give the details of the experiments conducted during the past three years.

#### DESCRIPTION OF EXPERIMENTS.

*Strain A.*—Several strains of *proteus* bacilli have been used. Strain A was obtained from a suppurating laparotomy wound through which a tuberculous kidney had been removed. The following twenty-six experiments were made with this strain.

*Experiment I.*<sup>1</sup>—Rabbit. Apr. 29, 1912. Inoculated intraperitoneally with one-fourth of a twenty-four hour slant agar culture obtained directly from the laparotomy wound. May 11. Death. Loss of weight, 25 per cent. At autopsy numerous soft, whitish nodules were found on the mesentery; also a few on the diaphragm and posterior abdominal wall. The lesions were in the peritoneum and subjacent tissue.

*Microscopic Examination.*—The nodules are composed mainly of a mass of polymorphonuclear leucocytes surrounded by a well developed wall of young connective tissue. There is necrosis in the central part of some of the abscesses. *Bacillus proteus* was recovered in pure culture from the lesions.

<sup>1</sup> These serial numbers are used for convenient reference. They do not always indicate the exact order in which the experiments were performed.

*Experiment 2.*—Rabbit. May 13, 1912. Inoculated intraperitoneally with a loopful of a twenty-four hour agar culture from experiment 1. May 30. Killed. Loss of weight, 11 per cent. At autopsy a number of small, whitish nodules were found on the omentum, mesentery, and liver.

*Microscopic Examination.*—The nodules are composed mainly of large connective tissue cells of the so called epithelioid type (figures 1 to 4). This is a typical proliferative inflammation. In histological structure it resembles an epithelioid cell tubercle. It will be described more fully below. One of the chief objects of our subsequent experiments with *B. proteus* was the study of this peculiar proliferative lesion.

*Experiment 3.*—Rabbit. May 20, 1912. Inoculated intravenously with 1 c.c. of a forty-eight hour broth culture from experiment 1. This broth culture was taken from an agar culture seven days old. May 27. Killed. Loss of weight, 10 per cent. At autopsy a large number of whitish nodules, some attaining a diameter of 5 mm., were seen in the liver. The lesions are scattered through the parenchyma of the organ. Similar nodules are found in both kidneys. The right lung contains large abscesses which have destroyed a considerable part of the organ.

*Microscopic Examination.*—The lesions are ordinary abscesses, consisting mainly of polymorphonuclear leucocytes. There is very little tendency toward encapsulation of the abscesses. *B. proteus* was obtained in pure culture from the lesions. This was the only instance in which we succeeded in producing macroscopic lesions by intravenous inoculation.

*Experiment 4.*—Rabbit. May 20, 1912. Inoculated intraperitoneally with 1 c.c. of the same culture used in experiment 3. May 25. Killed. Loss of weight, 19 per cent. At autopsy the omentum, mesentery, and the abdominal surface of the diaphragm were studded with small whitish nodules.

*Microscopic Examination.*—The nodules are composed of large connective tissue cells of epithelioid type, among which are a variable number of large and small mononuclear leucocytes. In some nodules the leucocytes predominate. No polymorphonuclear leucocytes are present. The lesions are, therefore, partly of proliferative and partly of exudative character. *B. proteus* was recovered in pure culture.

It will be noted that the original culture produced abscesses only (experiment 1). Cultures from experiment 1, injected intraperitoneally, produced proliferative lesions in experiment 2, and mixed exudative and proliferative lesions in experiment 4. When injected intravenously, the culture from experiment 1 produced abscesses (experiment 3).

*Experiment 5.*—Rabbit. June 7, 1912. Inoculated intraperitoneally with one-half of a twenty-four hour agar culture, taken from an agar culture which had stood in the laboratory since Apr. 16. No symptoms of infection appeared. June 13. Killed. Increase in weight, 1 per cent. No lesions were found at autopsy.

*Experiment 6.*—Rat. June 11, 1912. Inoculated intraperitoneally with 3 c.c.

of an eighteen hour broth culture, taken from the agar culture which had stood in the laboratory since Apr. 16. No symptoms of infection developed. June 17. Killed. Loss of weight, 10 per cent. No lesions were found at autopsy.

*Experiment 7.*—Rabbit. June 13, 1912. Inoculated intravenously with the same culture used in experiment 5. June 19. Killed. Loss of weight, 2 per cent. No lesions were found at autopsy.

These three experiments (5, 6, and 7) show that strain A had lost its virulence entirely by being kept in the laboratory for about two months.

*Experiment 8.*—Rat. June 11, 1912. 3 c.c. of an eighteen hour broth culture from experiment 4 were injected into the peritoneal cavity. Symptoms of infection developed and gradually became more severe. June 17. Animal moribund. Killed. Loss of weight, 29 per cent. At autopsy yellowish white nodules were found on the omentum, diaphragm, and liver.

*Microscopic Examination.*—Not made, but *B. proteus* was recovered in pure culture from the lesions.

*Experiment 9.*—Guinea pig. June 13, 1912. Inoculated intraperitoneally with 2 c.c. of a twenty-four hour broth culture from experiment 4, taken May 25. June 19. Killed. Loss of weight, 17 per cent. Large whitish nodules were found in the omentum at autopsy.

*Microscopic Examination.*—The nodules consist of epithelioid cells among which are a considerable number of polymorphonuclear leucocytes. The lesions represent a mixture of the exudative and the proliferative types of inflammatory reaction. They resemble the lesions found in experiment 4, except that the leucocytes in this instance are of the polymorphonuclear type.

*Experiment 10.*—Rat. June 19, 1912. 4 c.c. of a twenty-four hour broth culture from experiment 8 were given intraperitoneally. Death occurred within a few hours. The organism was recovered from the peritoneal cavity.

*Experiment 11.*—Rat. June 19, 1912. 3 c.c. of the same culture used in experiment 10 were injected. Death occurred the following night. The organism was recovered from the peritoneal cavity.

*Experiment 12.*—Rat. June 19, 1912. Intraperitoneal injection with 2 c.c. of the same culture used in experiment 10. Animal was sick the following day, but recovered. Increase in weight, 15 per cent. June 24. Killed. No lesions were found at autopsy.

*Experiment 13.*—Rat. June 19, 1912. 1 c.c. of the same culture used in experiment 10 was injected. No symptoms developed. Increase in weight, 5 per cent. June 24. Killed. No lesions found.

*Experiment 14.*—Rats. June 20, 1912. Three rats were each inoculated with 1.5 c.c. of a broth culture from experiment 8. All died the following night. The organism was recovered from the peritoneal cavity.

The preceding five experiments show that a *proteus* strain which had recently produced lesions in rats may lose this property entirely, although it may still be toxic when given in large doses.

*Experiment 15.*—Rats. June 21, 1912. Three rats were each inoculated intraperitoneally with 1 c.c. of a seven hour broth culture from experiment 14. All three were found dead the next morning. The organism was recovered from the peritoneal cavity.

*Experiment 16.*—Rats. June 22, 1912. Four rats were each injected intraperitoneally with 0.25 c.c. of a twenty-four hour broth culture from experiment 14. The first three died the following night. The organism was recovered from the peritoneal cavity. The fourth was sick the next day but recovered. No lesions were found when it was killed nine days later.

Experiments 15 and 16 show that the toxicity of strain A for rats had been markedly increased by a series of animal passages.

*Experiment 17.*—Rats. June 24, 1912. Two rats were injected intraperitoneally with 1 c.c. of a washed broth culture of *proteus* from experiment 16. No symptoms of infection appeared. Both were killed seven days later. No lesions were found at autopsy.

It appears from this experiment that the toxic properties of this *proteus* strain bear no relation to its ability to produce lesions. This is the same strain that produced lesions in experiment 8. By a series of animal passages the toxicity became markedly exalted, but, when the toxins were removed, the organism was harmless.

*Experiment 18.*—Rabbit. Aug. 2, 1912. Injected intravenously with one-fourth of an agar culture from experiment 9, which had stood in the laboratory since June 19. Aug. 8. Animal was in good condition. Aug. 22. Death from an intercurrent infection. No *proteus* lesions were found at autopsy.

*Experiment 19.*—Rabbit. Aug. 16, 1912. Inoculated intraperitoneally with one-half of a twenty-four hour culture of the same organism used in experiment 18. The animal gained in weight for several days. Death from pneumonia. Nov. 3. No *proteus* lesions were found at autopsy.

Experiments 18 and 19 demonstrate that strain A had lost its virulence entirely. No further experiments were made until April, 1913. The *proteus* cultures were kept in the laboratory during the intervening period.

*Experiment 20.*—Rabbits. One of the old cultures from strain A, which had long since lost its virulence, was injected into the anterior chamber of the eye of a rabbit (experiment 20 a). Twenty-four hours later it was recovered and this culture was injected into the peritoneal cavity of another rabbit (experiment 20 b), Apr. 26, 1913. The latter died the next day. *B. proteus* was recovered in pure culture from the peritoneal cavity and heart's blood.

*Experiment 21.*—Rabbit. Apr. 27, 1913. Injected intravenously with the same culture that was used intraperitoneally in experiment 20 b. No symptoms of infection developed.

*Experiment 22.*—Rabbit. Apr. 28, 1913. Intraperitoneal injection with one-

half of an agar culture from experiment 20 b. May 3. Animal was moribund. Killed. Loss of weight, 35 per cent. The omentum, both surfaces of the diaphragm, and the parietal pericardium were studded with soft whitish nodules. There were also a few nodules on the intestines. Emaciation was extreme.

*Microscopic Examination.*—The nodules are composed mainly of polymorphonuclear leucocytes. The central portions are necrotic. *B. proteus* was recovered in pure culture.

*Experiment 23.*—Rabbit. Apr. 28, 1913. Injected intraperitoneally with one-half of an agar culture from experiment 20 b. The animal lost weight for a few days, but began to regain it. May 6. Killed. Large, yellowish white nodules were found in the omentum, mesocolon, and parietal peritoneum.

*Microscopic Examination.*—The lesions consist of masses of polymorphonuclear leucocytes surrounded by a zone of newly formed connective tissue. They are healing abscesses. *B. proteus* was recovered in pure culture.

*Experiment 24.*—Rabbits. May 5, 1913. Two rabbits were each injected intraperitoneally with one loop of an agar culture from experiment 22. No symptoms of infection developed.

*Experiment 25.*—Rabbit. May 7, 1913. Inoculated subcutaneously with one-half of an agar culture from experiment 23. May 9. Death from an intercurrent infection. A small abscess was found at the site of the injection, from which *B. proteus* was recovered in pure culture. Heart's blood negative.

*Experiment 26.*—Rabbit. May 7, 1913. Inoculated subcutaneously with an old culture of strain A which had been made virulent by passage through the anterior chamber of the eye of a rabbit, as in experiment 20 a. May 15. Killed. Loss of weight, 41 per cent. A large subcutaneous abscess was found at the site of the inoculation, from which *B. proteus* was recovered in pure culture.

Experiments 20 to 26 show that an avirulent *proteus* strain can be made virulent by inoculation into the anterior chamber of the eye. It is also seen that strain A does not now produce proliferative lesions.

*Strain B.*—This strain was isolated from an eye infection, following a cataract operation which necessitated removal of the eyeball.

*Experiment 27.*—Rabbit. May 28, 1912. Injected intraperitoneally with 1 c.c. of a twenty-four hour broth culture of this organism. Death occurred on June 4. Loss of weight, 16 per cent. Autopsy revealed several rather large abscesses in the omentum, from which *B. proteus* was recovered in pure culture.

*Microscopic Examination.*—The nodules were found to be typical abscesses.

*Strain C.*<sup>25</sup>—Strain C was obtained from an infection of a finger, which was contracted while assisting at an autopsy.

*Experiment 28.*—Rabbit. July 29, 1913. Injected intraperitoneally with this culture. No symptoms of infection developed. Aug. 11. Laparotomy revealed a large number of peritoneal lesions. There had been only a slight loss of weight. Aug. 15. Killed. At autopsy a number of small whitish nodules were found on the omentum and colon.

*Microscopic Examination.*—The nodules showed a proliferative lesion similar to that described in experiment 2.

*Experiment 29.*—Rabbit. Aug. 18, 1913. Injected intraperitoneally with one-half of a five hour agar culture from experiment 28. Death occurred the following night.

*Experiment 30.*—Rabbit. Aug. 19, 1913. Injected intraperitoneally with a culture from experiment 28. Animal became ill and lost nearly 200 gm. in weight, but gradually recovered.

*Experiment 31.*—Rabbit. Sept. 4, 1913. Injected intraperitoneally with a culture from experiment 28. No symptoms of infection developed.

*Experiment 32.*—Rabbit. Sept. 26, 1913. Injected subcutaneously and intraperitoneally with a culture from experiment 28. No symptoms of infection appeared.

*Experiment 33.*—Rabbit. Sept. 26, 1913. Injected intraperitoneally with a culture from experiment 28. Sept. 30. Animal dying of an intercurrent disease. Killed. No lesions were found in the peritoneal cavity.

Experiment 28 shows that strain C had the power to produce lesions in rabbits when it was first recovered from human tissue. Experiments 31 to 33 show that this property rapidly disappeared when the organisms were grown in the laboratory.

*Strain D.*—Strain D was obtained from the heart's blood in a human autopsy. The main autopsy findings were a gunshot wound through the abdomen, producing multiple perforations of the intestines, general peritonitis, and lobar pneumonia. *Bacillus proteus* was also isolated from the peritoneal cavity.

*Experiment 34.*—Rabbit. Sept. 23, 1913. Injected intraperitoneally with one-half of a twenty-four hour agar culture of strain D. Sept. 24. Died. The peritoneal cavity contained a considerable amount of thin blood-stained fluid. *B. proteus* was recovered from this fluid. The plates contained an occasional staphylococcus colony.

*Experiment 35.*—Rabbit. Sept. 24, 1913. Injected intraperitoneally with a small dose of the culture used in experiment 34. Sept. 29. Killed. No lesions were found at autopsy.

*Experiment 36.*—Rabbit. Sept. 24, 1913. Inoculated subcutaneously with one-half of a five hour agar culture from experiment 34. Death occurred during the night.

*Experiment 37.*—Rabbit. Oct. 8, 1913. Injected subcutaneously with ag-gressin from a culture of strain D. Two hours later the rabbit was inoculated subcutaneously with a loopful of a culture of the same strain. Symptoms of severe infection appeared. A large subcutaneous abscess developed at the site of the inoculation. Oct. 14. The abdominal cavity was accidentally opened while exploring the abscess. Oct. 16. Death occurred from peritonitis. Loss of weight, 21 per cent. *B. proteus* was recovered in pure culture from the peritoneum as well as from the abscess.

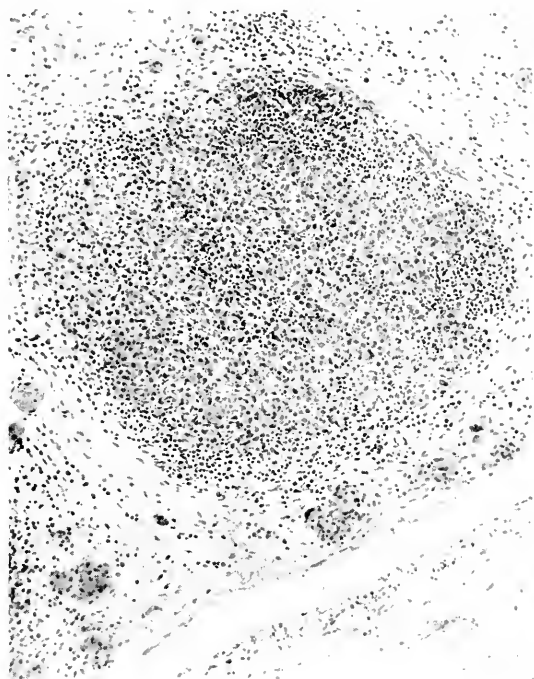


FIG. 1.

(Larson and Bell: Properties of *Bacillus proteus*.)





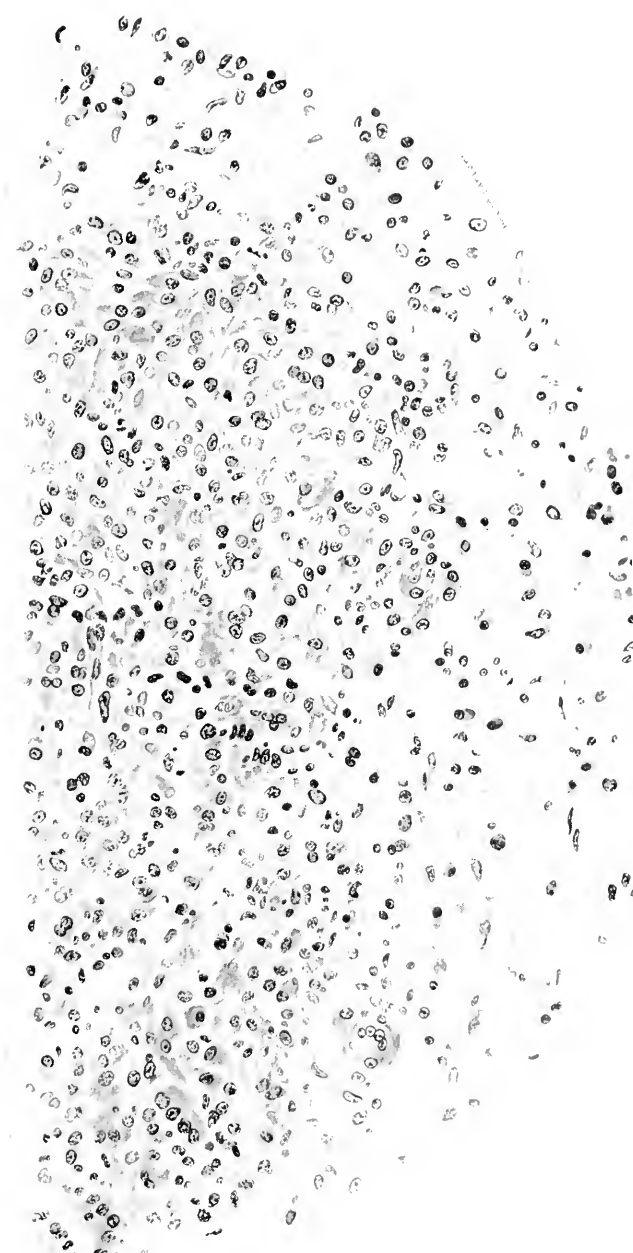


FIG. 2.

(Larson and Bell: Properties of *Bacillus proteus*.)



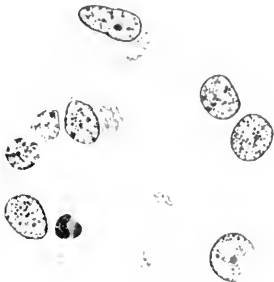


FIG. 3.



FIG. 4.

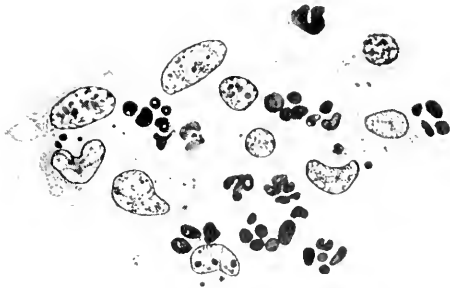


FIG. 5.

(Larson and Bell: Properties of *Bacillus proteus*.)



*Experiment 38.*—Rabbit. Oct. 8, 1913. Injected subcutaneously with ag-gressin from a culture of strain D. One hour later inoculated intraperitoneally with a loopful of a culture of the same strain. A large subcutaneous abscess developed at the site of the inoculation. Loss of weight in eight days, 22 per cent. After about four weeks the abscess began to disappear. Dec. 17. Death from an intercurrent disease. The abscess had disappeared entirely by this time. There were no *proteus* lesions present.

*Experiment 39.*—Rabbit. Oct. 15, 1913. Inoculated intraperitoneally with one-half of a twenty-four hour agar culture taken from experiment 37 at operation. Oct. 24. Died. Loss of weight, 30 per cent. Autopsy revealed a considerable number of whitish nodules, 1 mm. to 3 mm. in diameter, on the mesentery, omentum, diaphragm, and posterior abdominal wall.

*Microscopic Examination.*—Most of the nodules are ordinary abscesses; but a few show the proliferative type of lesion.

*Strain E.*—Strain E was obtained from a gangrenous human lung.

*Experiment 40.*—Rabbit. Dec. 3, 1913. Inoculated intraperitoneally with a twenty-four hour culture of this organism. Dec. 8. Died. At autopsy a large number of characteristic nodules were found on the peritoneal surfaces. Microscopically, some of the nodules were found to be ordinary abscesses. Other nodules show proliferative inflammation. *B. proteus* was recovered from the lesions.

*Experiment 41.*—Rabbit. Dec. 10, 1913. Inoculated intraperitoneally with one-half of an agar culture from experiment 40. Dec. 18. Killed. Loss of weight, 13 per cent. Numerous soft, whitish nodules were found on the peritoneal surfaces.

*Microscopic Examination.*—Some of the nodules are ordinary abscesses surrounded by a well developed wall of fibroblasts. Other nodules show mainly the proliferative lesion. There are masses of large connective tissue cells of the epithelioid type among which a considerable number of polymorphonuclear leucocytes are interspersed (figure 5).

*Experiment 42.*—Rabbit. Dec. 19, 1913. Inoculated intravenously with one-half of an agar culture from experiment 41. Died the next day. *Proteus* recovered from the heart's blood.

*Experiment 43.*—Rabbit. Dec. 23, 1913. Inoculated intraperitoneally with one-half of an agar culture from experiment 42. Dec. 30. Laparotomy showed no lesions in the peritoneal cavity.

*Experiment 44.*—Rabbit. Dec. 25, 1913. Injected intravenously with one-half of an agar culture from experiment 42. No symptoms of infection developed.

The proliferative lesion appeared with strain E in experiment 40, and again in experiment 41. It was not followed further, since the strain suddenly lost its virulence.

#### DISCUSSION.

From the above experiments it will be seen that some strains of *Bacillus proteus* obtained from human lesions are definitely patho-

genic for rabbits, rats, and guinea pigs, producing either typical abscesses or a granulomatous type of lesion. Not all strains of the *proteus* are pathogenic, however. During the past three years we have experimented with twenty other strains which were obtained from various sources, such as decaying organic material and routine autopsy blood cultures where they were probably merely postmortem invaders. In one case a non-pathogenic strain was isolated from an amebic abscess of the liver. None of these proved to be pathogenic for laboratory animals. Rabbits injected either intravenously or intraperitoneally with one to two cubic centimeters of broth cultures of such strains suffered little or no inconvenience. Where excessive doses were given the animals died with symptoms of acute toxemia.

The five strains of *proteus* which were pathogenic for laboratory animals were obtained from human lesions from which, with one exception (strain E), no other organisms were recovered. It is, therefore, probable that these organisms were responsible for the lesions in which they were found. Only in two cases (strains C and E) was it possible to examine microscopically the tissues from which the organisms were obtained. Sections from the infected finger (strain C) showed atypical tubercles, but repeated examinations failed to reveal acid-fast bacilli. However, the possibility of a tuberculous infection in this case cannot be excluded. Strain E was obtained from a gangrenous lung in association with pneumococci. No cellular structure was visible in the area from which the culture was taken. Outside the gangrenous area the lung showed typical lobar pneumonia.

From our work covering a study of twenty-five different *proteus* strains we have come to regard a positive rabbit inoculation as strong evidence that the strain is pathogenic for man. It must be borne in mind that a positive inoculation does not necessarily cause the death of the animal, since some animals may recover from rather severe infections. The method of inoculation is very important. The intraperitoneal inoculation is the method of choice, since intraperitoneal infections are more severe than subcutaneous infections. Inoculations made intravenously rarely result in the production of the characteristic lesions.

The toxic properties of the *proteus* bacilli must not be confused with their power of producing the characteristic inflammatory reactions described in our experiments; in fact, these two characters bear no relation to each other. As indicated in experiments 10 to 16, the toxicity of the organism may become most markedly exalted, while its power to produce the lesions is entirely lost.

*Proteus* cultures lose their virulence rapidly when kept on laboratory media. The original culture of strain A, which was at first very virulent, became non-pathogenic in about two months (experiments 5, 6, and 7). Another culture of this strain lost its virulence in six weeks (experiments 18 and 19). Strain C became avirulent in three weeks (experiment 31); and strain E in less than one week (experiments 43 and 44). This rapid loss of virulence may explain the fact that *Bacillus proteus* is seldom regarded by bacteriologists as an important pathogenic organism.

A non-pathogenic *proteus* may be made pathogenic by inoculation into the anterior chamber of the eye. In this situation the organisms grow rapidly, there being neither antibodies nor complement present. If recovered from the anterior chamber after twenty-four hours, the culture will usually be found to be pathogenic (experiments 20, 22, and 23). Non-pathogenic strains of streptococci may also become very virulent for rabbits when grown in the anterior chamber, as just described.

A non-pathogenic *proteus* may be enabled to produce lesions by the use of aggressins. The aggressin is injected one or two hours before the animal is inoculated with the bacteria (experiments 37 and 38).

Strains of *proteus*, made pathogenic by either of the above described procedures, produce lesions similar to those produced by strains that were pathogenic when obtained from human lesions.

#### SYMPTOMS OF PROTEUS INFECTION IN LABORATORY ANIMALS.

The clinical symptoms of experimental *proteus* infection in animals present marked variations, depending mainly upon the virulence of the bacteria. In the mildest cases the animals may not show any signs of infection. In one case (experiment 28), which presented no clinical symptoms, laparotomy revealed rather extensive peritoneal lesions. Mild cases show a temporary loss of weight, weakness, and

decreased appetite, after which they gradually recover. Occasionally an animal will recover from a very severe infection (experiment 38).

Several of the rabbits that were killed might otherwise have recovered from the *proteus* infection.

In very severe *proteus* infections the animals become extremely emaciated. In one case a loss of weight of 41 per cent. was recorded. Losses of 20 to 30 per cent. are common. Emaciation is a striking feature of severe *proteus* infections. The animals refuse to eat and become progressively weaker. Death usually results in about one week. Deaths from toxemia, without the production of lesions, usually occur within the first twenty-four hours.

#### GROSS AND MICROSCOPIC PATHOLOGY.

The characteristic lesions resulting from intraperitoneal inoculation are fairly firm, whitish nodules, one to five millimeters in diameter, sometimes larger. They may occur on any part of the peritoneal surface. They were most frequently found in the omentum in our experiments, apparently because the injecting needle came into contact with this structure. The number and size of the lesions usually corresponded to the clinical severity of the infection. In one case the infection passed through the diaphragm and involved the lungs and pleuræ. In no other instance did the lesions resulting from an intraperitoneal inoculation extend beyond the peritoneal cavity.

The large nodules with softened centers always show the exudative type of lesion histologically; but, as a rule, the exudative and proliferative lesions cannot be distinguished by macroscopic examination. When subcutaneous inoculation is successful it usually results in the formation of an abscess of varying size. Death may result from a subcutaneous lesion; but an intraperitoneal infection advances more rapidly and is more apt to terminate fatally.

By intravenous inoculation we succeeded in producing the characteristic lesions in only one case (experiment 3). In this instance there were abscesses in the liver, both kidneys, and the right lung. *Bacillus proteus* disappears rapidly from the blood stream. After intravenous inoculation it was recovered only in those cases that died within the first twenty-four hours. It was never recovered from



the heart's blood after subcutaneous inoculations, and only once after intraperitoneal injection.

As regards histological structure the *proteus* lesions show an exudative or a proliferative type of inflammation, or a mixture of these two forms of tissue reaction. The exudative lesions occur more frequently. They are simple abscesses composed mainly of polymorphonuclear leucocytes. If the infection is not too severe, the abscesses become walled off by a zone of newly formed connective tissue. In the one case in which intravenous inoculation was successful, typical abscesses were formed. The subcutaneous lesions in every case were of the exudative type. The exudative lesions have no special histological interest since they are ordinary abscesses.

The proliferative lesions, however, demand particular attention. Grossly, the nodules are usually smaller and firmer than the abscesses, but it is often not possible to distinguish them from the exudative lesions by gross examination. Figure 1 represents a small nodule of the proliferative type under low magnification. It consists mainly of large cells resembling the so called epithelioid cells of a tubercle. This lesion may indeed be grouped with the granulomata. Masses of small lymphocytes are seen around the periphery of the nodule and in certain places within it. Below the large nodule are seen several smaller ones which consist of a few epithelioid cells. In a section through one of the macroscopic omental nodules of this case, one sees several masses resembling in structure the one shown in figure 1, but some of them are many times larger.

A higher magnification of a part of a proliferative lesion is shown in figure 2. There is a gradual transition from the dense part of the nodule to the adjacent fibrous connective tissue. The cytoplasm of some of the epithelioid cells fuses with that of adjacent cells. Blood vessels are not present in the denser parts of the lesion.

Figures 3 and 4 represent small areas from figure 2 under very high magnification. Multinucleated cells are shown in figure 3. The epithelioid cells are frequently found fused, in this way forming giant-cells, but no giant-cells of the Langhans type have ever been observed in any case. Figure 4 represents an area in which the cells are all distinct. In both figures delicate connective tissue fibrillae are shown running between the cells and apparently con-

nected to their cytoplasm. By means of Mallory's phosphotungstic hematoxylin stain, it is easy to see that these fibrillæ are present everywhere among the epithelioid cells.

The smallest recognizable nodules, such as are shown in the lower part of figure 1, consist of a few epithelioid cells. The delicate fibrillæ are always present except where the cytoplasm of the cells is fused. Careful examination of the peripheral parts of the nodules (figure 2) will show that there is a gradual transition between the epithelioid cells and the adjacent fixed connective tissue cells. There is no evidence that the epithelioid cells are derived from the endothelium of blood or lymph vessels. All the evidence available indicates that they develop from the fixed cells of the connective tissue. The black masses shown in the cytoplasm of the cells in figures 3 and 4 are fragments of the nuclei of leucocytes. In some proliferative lesions all the epithelioid cells are crowded with coarse, deeply stained nuclear fragments. These cells readily ingest the leucocytes that enter the lesion.

Frequently the nodules consist of epithelioid cells among which are numerous polymorphonuclear leucocytes. Such a lesion is shown in figure 5. This may be regarded as a mixture of the exudative and proliferative types of inflammation. As in figure 5, the leucocytes in these lesions usually show pronounced signs of degeneration. They evidently degenerate readily and are ingested by the epithelioid cells.

Sections appropriately stained<sup>2</sup> show enormous numbers of bacilli everywhere in the proliferative lesion. The bacilli all seem to be extracellular in position.

There seems to be no relation between the clinical severity of the case and the histological type of the lesion. Neither does the histology of the lesion depend upon the particular strain of *proteus* used, since all types of lesions were produced at different times with strain A. The virulence of the particular culture employed, though it may be of importance, is not the only factor, since both exudative and proliferative lesions were sometimes found close together in

<sup>2</sup> To stain the bacilli the tissues should be fixed in Zenker's fluid, sectioned in paraffin, and stained by Mallory's eosin-methylene blue method (overstaining with the methylene blue). This method was used by Mallory to show the Bordet-Gengou bacillus in tissue sections.

the same experiment (experiments 39, 40, and 41). We did not discover the factors that control the histology of the lesion.

#### SUMMARY.

Some strains of *Bacillus proteus* obtained from human lesions are pathogenic for rabbits, rats, and guinea pigs. There is good evidence that these strains are also pathogenic for man.

All cultures obtained from sources other than human infections were non-pathogenic for the laboratory animals.

A non-pathogenic culture may be made pathogenic by the use of aggressins or by inoculation into the anterior chamber of the eye.

*Proteus* cultures lose their virulence rapidly when grown on artificial media.

The lesions produced in animals are either simple abscesses, proliferative lesions, or a mixed exudative and proliferative lesion.

The proliferative lesions consist mainly of epithelioid cells apparently of connective tissue origin. No giant-cells of the Langhans type are present.

The histological type of the lesion does not depend upon the strain employed. Neither does it bear any relation to the clinical severity of the case.

The ability to produce the characteristic lesions has no necessary connection with the toxicity of the bacteria.

The *proteus* bacteria probably play a more important part in human pathology than is generally believed.

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## EXPLANATION OF PLATES.

## PLATE 65.

FIG. 1. Experiment 2. Proliferative lesion from the omentum. Several very small nests of epithelioid cells are shown in the lower part of the figure. Microphotograph.  $\times 120$ .

## PLATE 66.

FIG. 2. Experiment 2. Proliferative lesion. One-half of a nodule similar to the one shown in figure 1.  $\times 350$ .

## PLATE 67.

FIG. 3. Experiment 2. Small area from figure 2. Note the fusion of the epithelioid cells. Delicate connective tissue fibrillæ are shown. The black mass in the cytoplasm is a pycnotic nucleus.  $\times 900$ .

FIG. 4. Experiment 2. Small area from figure 2. Connective tissue fibrillæ are prominent. A disintegrating leucocyte is shown in the cytoplasm of one of the cells.  $\times 900$ .

FIG. 5. Experiment 41. Mixed exudative and proliferative lesion. The polymorphonuclear leucocytes show various stages of degeneration.  $\times 900$ .

## INDEX TO VOLUME XXI.

- ABDERHALDEN** reaction, mechanism of, 221
- Abderhalden reaction, mechanism of, and serum proteases, 239
- Absorption, subcutaneous, and quantitative estimation of cholesterol, 179
- Agglutination—antibody formation against *Treponema pallidum*, 576
- Alcohol, phosphorus, or chloroform, hepatic lesions of, and diet, 1
- ALFORD, LELAND B. See OPIE and ALFORD, I, 21
- AMOSS, HAROLD L. See FLEXNER and AMOSS, 509, 515
- , See FLEXNER, NOGUCHI, and AMOSS, 91
- Anaphylatoxin, nature of, 480
- Anemia, experimental aplastic, of benzol poisoning, factors of coagulation in, with special reference to origin of prothrombin, 401
- Anesthetics and narcotics, liver function as influenced by, 203
- Animal tissues and bacteria under conditions of artificial cultivation, reactions between, 103
- Anterior lobe of pituitary body, influence of, upon growth of carcinomata, 280
- Antibody formation against *Treponema pallidum*—agglutination, 576
- Antidysenteric serum, rapid production of, 515
- Antipneumococcus serum, concentration of protective bodies in, by specific precipitation, 389
- Antipneumococcus serum, distribution of immune bodies occurring in, 133
- Antithrombin, test for, in blood, 338
- Aplastic anemia, experimental, of benzol poisoning, factors of coagulation in, with special reference to origin of prothrombin, 401
- Arsenic compounds, effect of, on Rous chicken sarcoma, 574
- Artificial cultivation, reactions between bacteria and animal tissues under conditions of, 103
- Atoxyl and paraminophenylarsenoxyl, experiments *in vitro* with, influence of serum upon trypanosomes with special reference to its use for, 250
- Atoxyl, transformed, different amounts produced of, by incubating 1 per cent. and 10 per cent. atoxyl in blood, 258
- AUER, JOHN. The functional effect of experimental intraspinal injections of sera with and without preservatives, 43
- Autoplastic and homeoplastic transplantation of thyroid tissue in the guinea pig, comparison of, 164
- AVERY, OSWALD T. The distribution of the immune bodies occurring in antipneumococcus serum, 133
- , See DOCHEZ and AVERY, 114
- BACILLI**, tubercle, new and rapid method for isolation and cultivation of, directly from sputum and feces, 38
- Bacillus proteus*, pathogenic properties of, 629
- Bacillus tuberculosis*, experimental infection of albino mice with, function of spleen in, 84
- Bacteria and animal tissues under conditions of artificial cultivation, reactions between, 103
- Bacteria, pure cultivation *in vivo* of vaccine virus free from, 539
- BAITSELL, GEORGE A. The origin and structure of a fibrous tissue which appears in living cultures of adult frog tissues, 455
- BARBOUR, HENRY G., and PRINCE, ALEXANDER L. The influence of epinephrin upon the coronary circulation of the monkey, 330
- Barlow's disease experimentally produced in fetal and new-born guinea pigs, 525
- BELL, E. T. See LARSON and BELL, 629
- Benzol poisoning, factors of coagulation in experimental aplastic anemia of, with special reference to origin of prothrombin, 401
- Biological properties of *Trypanosoma lewisi*, changes in, produced by ex-

- perimental means, with special reference to virulence, 345
- Blood, different amounts of transformed atoxyl produced by incubating 1 per cent. and 10 per cent. atoxyl in, 258
- Blood, effect of heat on transforming and binding power of, 267
- Blood, test for antithrombin in, 338
- BRONFENBRENNER, J. The mechanism of the Abderhalden reaction. Studies on immunity. I, 221
- The nature of anaphylatoxin. Studies on immunity. II, 480
- BROWN, WADE H. Concerning changes in the biological properties of *Trypanosoma lewisi* produced by experimental means, with especial reference to virulence, 345
- BURNETT, THEODORE C. See ROBERTSON and BURNETT, 280
- CARCINOMATA, influence of anterior lobe of pituitary body upon growth of, 280
- Cells, epithelial, normal and regenerating, of the rat and guinea pig, proliferative power of, 193
- CHICKERING, HENRY T. See GAY and CHICKERING, 389
- Chloroform, phosphorus, or alcohol, hepatic lesions of, and diet, 1
- Chloroform poisoning, late, resistance of pups to, in its relation to liver glycogen, 185
- Chloroform, potassium chromate, or uranium nitrate, nephritis caused by, and diet, 21
- Cholesterol, subcutaneous absorption and quantitative estimation of, 179
- Chromate, potassium, uranium nitrate, or chloroform, nephritis caused by, and diet, 21
- Chronic uranium nephritis, histogenesis of, with especial reference to epithelial regeneration, 425
- Circulation, coronary, of monkey, influence of epinephrin on, 330
- Circulation in the pneumonic lung, and *intra vitam* staining in experimental pneumonia, 311
- Coagulation in experimental aplastic anemia of benzol poisoning, factors of, with special reference to origin of prothrombin, 401
- COHN, ALFRED E., FRASER, FRANCIS R., and JAMIESON, ROSS A. The influence of digitalis on the T wave of the human electrocardiogram, 593
- Coronary circulation of monkey, influence of epinephrin on, 330
- CORPER, HARRY J. Notes on the subcutaneous absorption and the quantitative estimation of cholesterol, 179
- Corpuscles, red, extracellular relation of the parasites to. Adult tertian malarial parasites attached to peripheral corpuscular mounds, 584
- Cortex, visual, its histological structure, localization, and physiological function, 617
- Cultivated microorganism from poliomyelitic tissues, survival and virulence of, 91
- Cultivation and isolation of tubercle bacilli directly from sputum and feces, new and rapid method for, 38
- Cultivation, artificial, reactions between bacteria and animal tissues under conditions of, 103
- Cultivation of *Treponema pallidum*, 213
- Cultivation, pure, *in vivo*, of vaccine virus free from bacteria, 539
- Cultures, living, of adult frog tissues, origin and structure of a fibrous tissue which appears in, 455
- DIET and the hepatic lesions of chloroform, phosphorus, or alcohol, 1
- Diet and the nephritis caused by potassium chromate, uranium nitrate, or chloroform, 21
- Diet, influence of, upon necrosis caused by hepatic and renal poisons, 1, 21
- Diffusion and survival of poliomyelitic virus, 509
- Digitalis, influence of, on T wave of human electrocardiogram, 593
- Diphtheroids, wide-spread distribution of, and their occurrence in various lesions of human tissues, 493
- DOCHEZ, A. R., and AVERY, O. T. Varieties of pneumococcus and their relation to lobar pneumonia, 114
- DRINKER, C. K. See HURWITZ and DRINKER, 401
- EGGSTAIN, A. A. See JOBLING, EGGSTAIN, and PETERSEN, 239
- Electrocardiogram, human, influence of digitalis on T wave of, 593
- Epidermis of the rat and guinea pig, relation between structure of, and proliferative power of normal and regenerating epithelial cells of the same species, 193

- Epinephrin, influence of, on coronary circulation of monkey, 330
- Epithelial cells, normal and regenerating, of the rat and guinea pig, proliferative power of, 193
- Epithelial regeneration in reference to histogenesis of chronic uranium nephritis, 425
- Estimation, quantitative, and subcutaneous absorption of cholesterol, 179
- Etiology of goitre in brook trout. IV. Effect of feeding with fresh and stale liver, 452
- Experimental aplastic anemia of benzol poisoning, factors of coagulation in, with special reference to origin of prothrombin, 401
- Experimental infection of albino mice with *Bacillus tuberculosis*, function of spleen in, 84
- Experimental intraspinal injections of sera with and without preservatives, functional effect of, 43
- Experimental means, changes in biological properties of *Trypanosoma lewisi* produced by, with special reference to virulence, 345
- Experimental pneumonia in rabbits, 304, 311, 320
- Experimental pneumonia in the rabbit, effect of sensitization on lesions of, 605
- Experimental pneumonia, *intra vitam* staining in, and the circulation in the pneumonic lung, 311
- Experimentally produced Barlow's disease in fetal and new-born guinea pigs, 525
- Experiments *in vitro* with atoxyl and paraminophenylarsenoxyl, influence of serum upon trypanosomes with special reference to its use for, 250
- Extracellular relation of the parasites to the red corpuscles. Adult tertian malarial parasites attached to peripheral corpuscular mounds, 584
- F**ECES and sputum, new and rapid method for isolation and cultivation of tubercle bacilli directly from, 38
- Ferment action, studies on, 239
- Fetal and new-born guinea pigs, Barlow's disease experimentally produced in, 525
- Fibrous tissue which appears in living cultures of adult frog tissues, origin and structure of, 455
- FLEISHER, MOYER S., and LOEB, LEO. Further investigations on the mode of action of substances inhibiting tumor growth and on immunization against these substances, 155
- FLEXNER, SIMON, and AMOSS, HAROLD L. Diffusion and survival of the poliomyelitic virus, 509
- The rapid production of anti-dysenteric serum, 515
- FLEXNER, SIMON, NOGUCHI, HIDEYO, and AMOSS, HAROLD L. Concerning survival and virulence of the microorganism cultivated from poliomyelitic tissues, 91
- Flora, intestinal, character of, mortality and growth, influence of milk feeding on, 365
- FRASER, FRANCIS R. See COHN.
- FRASER, and JAMIESON, 593
- FUNK, CASIMIR. The transplantation of tumors to foreign species, 571
- The effect of arsenic compounds on the Rous chicken sarcoma, 574
- FUNKHOUSER, EDGAR BRIGHT. The visual cortex, its localization, histological structure, and physiological function, 617
- G**AY, FREDERICK P., and CHICKERING, HENRY T. Concentration of the protective bodies in antipneumococcus serum by means of specific precipitation, 389
- GILBERT, RUTH. See ZINSSER, HOPKINS, and GILBERT, 213
- Glycogen, liver, resistance of pups to late chloroform poisoning in its relation to, 185
- Goitre in brook trout, etiology of. IV. Effect of feeding with fresh and stale liver, 452
- Gonococci, comparison of adult and infant types of, 289
- GRAHAM, EVARTS A. The resistance of pups to late chloroform poisoning in its relation to liver glycogen, 185
- Growth, mortality, and character of intestinal flora, influence of milk feeding on, 365
- Growth of carcinomata, influence of anterior lobe of pituitary body upon, 280
- Growth of tumors, mode of action of substances inhibiting, and immunization against these substances, 155
- H**ARRIS, WILLIAM H., and WADE, H. WINDSOR. The wide-spread distribution of diphtheroids and

- their occurrence in various lesions of human tissues, 493
- Heat, effect of, on transforming and binding power of blood, 267
- Hepatic and renal poisons, influence of diet upon necrosis caused by, 1, 21
- Hepatic lesions of chloroform, phosphorus, or alcohol, and diet, 1
- HESS, ALFRED F. A test for antithrombin in the blood, 338
- HESELBERG, CORA. A comparison of autoplasmic and homeoplastic transplantation of thyroid tissue in the guinea pig, 164
- Histogenesis of chronic uranium nephritis with especial reference to epithelial regeneration, 425
- Histological structure, localization, and physiological function of visual cortex, 617
- Homeoplastic and autoplasmic transplantation of thyroid tissue in the guinea pig, comparison of, 164
- HOPKINS, JOSEPH GARDNER. See ZINSSER and HOPKINS, 576
- . See ZINSSER, HOPKINS, and GILBERT, 213
- HURWITZ, S. H., and DRINKER, C. K. The factors of coagulation in the experimental aplastic anemia of benzol poisoning, with special reference to the origin of prothrombin, 401
- IMMUNE bodies occurring in anti-pneumococcus serum, distribution of, 133
- Immunity reaction, part of the leucocyte in, 320
- Immunity, studies on, 221, 480
- Immunization against substances inhibiting growth of tumors, and mode of action of these substances, 155
- In vitro* experiments with atoxyl and paraminophenylarsenoxyl, influence of serum upon trypanosomes with special reference to its use for, 250
- In vivo* pure cultivation of vaccine virus free from bacteria, 539
- Infection, experimental, of albino mice with *Bacillus tuberculosis*, function of spleen in, 84
- INGIER, ALEXANDRA. A study of Barlow's disease experimentally produced in fetal and new-born guinea pigs, 525
- Injectations, experimental intraspinal, of sera with and without preservatives, functional effect of, 43
- Intestinal flora, character of, mortality, and growth, influence of milk feeding on, 365
- Intra vitam* staining in experimental pneumonia, and the circulation in the pneumonic lung, 311
- Intraspinal injections, experimental, of sera with and without preservatives, functional effect of, 43
- JAMIESON, ROSS A. See COHN, FRASER, and JAMIESON, 593
- JOBLING, JAMES W., EGGSTEIN, A. A., and PETERSEN, WILLIAM. Serum proteases and the mechanism of the Abderhalden reaction. Studies on ferment action. XX, 239
- KIRKBRIDE, MARY BUTLER. A study of the effect of sensitization on the development of the lesions of experimental pneumonia in the rabbit, 605
- KLINE, B. S., and WINTERITZ, M. C. Studies upon experimental pneumonia in rabbits. VII. The production of lobar pneumonia, 304
- . Studies upon experimental pneumonia in rabbits. VIII. *Intra vitam* staining in experimental pneumonia, and the circulation in the pneumonic lung, 311
- KLINE, B. S. See WINTERITZ and KLINE, 320
- LARSON, W. P., and BELL, E. T. A study of the pathogenic properties of *Bacillus proteus*, 629
- LAWSON, MARY R. Adult tertian malarial parasites attached to peripheral corpuscular mounds. The extracellular relation of the parasites to the red corpuscles, 584
- Lesions, hepatic, of chloroform, phosphorus, or alcohol, and diet, 1
- Lesions of experimental pneumonia in the rabbit, effect of sensitization on, 605
- Lesions, various, of human tissues, wide-spread distribution of diphtheroids and their occurrence in, 493
- Leucocyte, part of, in immunity reaction, 320
- LEWIS, PAUL A., and MARGOT, ARTHUR GEORGES. The function of the spleen in the experimental infection of albino mice with *Bacillus tuberculosis*. Second paper, 84
- Lewis, *Trypanosoma*, changes in biological properties of, produced by



- experimental means, with special reference to virulence, 345
- Liver, fresh and stale, effect of feeding with, in relation to etiology of goitre in brook trout, 452
- Liver function as influenced by anesthetics and narcotics, 203
- Liver glycogen, resistance of pups to late chloroform poisoning in its relation to, 185
- Living cultures of adult frog tissues, origin and structure of a fibrous tissue which appears in, 455
- Lobar pneumonia, production of, in rabbits, 304
- Lobar pneumonia, relation of, to varieties of pneumococcus, 114
- Lobe, anterior, of pituitary body, influence of, upon growth of carcinomata, 280
- LOEB, LEO. See FLEISHER and LOEB, 155
- Lung, pneumonic, circulation in, and *intra vitam* staining in experimental pneumonia, 311
- LYALL, HAROLD W. The types of pneumococci in tuberculous sputum, 146
- M**ALARIAL parasites, adult tertian, attached to peripheral corpuscular mounds. Extracellular relation of the parasites to the red corpuscles, 584
- MARGOT, ARTHUR GEORGES. See LEWIS and MARGOT, 84
- MARINE, DAVID. Observations on the etiology of goitre in brook trout. IV. The effect of feeding with fresh and stale liver, 452
- Mechanism of Abderhalden reaction, 221
- Mechanism of Abderhalden reaction and serum proteases, 239
- Microorganism cultivated from poliomyelitic tissues, survival and virulence of, 91
- Milk feeding, influence of, on mortality and growth, and on character of intestinal flora, 365
- Mounds, peripheral corpuscular, adult tertian malarial parasites attached to. Extracellular relation of the parasites to the red corpuscles, 584
- N**ARCOTICS and anesthetics, liver function as influenced by, 203
- Necrosis caused by hepatic and renal poisons, influence of diet upon, 1, 21
- Nephritis caused by potassium chromate, uranium nitrate, or chloroform, and diet, 21
- Nephritis, chronic uranium, histogenesis of, with especial reference to epithelial regeneration, 425
- New-born and fetal guinea pigs, Barlow's disease experimentally produced in, 525
- Nitrate, uranium, potassium chromate, or chloroform, nephritis caused by, and diet, 21
- NOGUCHI, HIDEYO. Pure cultivation *in vivo* of vaccine virus free from bacteria, 539
- . See FLEXNER, NOGUCHI, and AMOSS, 91
- O**LIVER, JEAN. The histogenesis of chronic uranium nephritis with especial reference to epithelial regeneration, 425
- OPIE, EUGENE L., and ALFORD, LELAND B. The influence of diet upon necrosis caused by hepatic and renal poisons. Part I. Diet and the hepatic lesions of chloroform, phosphorus, or alcohol, 1
- . The influence of diet upon necrosis caused by hepatic and renal poisons. Part II. Diet and the nephritis caused by potassium chromate, uranium nitrate, or chloroform, 21
- P**ALLIDUM, *Treponema*, antibody formation against—agglutination, 576
- Pallidum*, *Treponema*, cultivation of, 213
- Paraminophenylarsenoxyl and atoxyl, experiments *in vitro* with, influence of serum upon trypanosomes with special reference to its use for, 250
- Parasites, adult tertian malarial, attached to peripheral corpuscular mounds. Extracellular relation of the parasites to the red corpuscles, 584
- Pathogenic properties of *Bacillus proteus*, 629
- PEARCE, LOUISE. A comparison of adult and infant types of gonococci, 289
- Peripheral corpuscular mounds, adult tertian malarial parasites attached to. Extracellular relation of the parasites to the red corpuscles, 584
- PETERSEN, WILLIAM. See JOBLING, EGSTEIN, and PETERSEN, 239

- PETROFF, S. A. A new and rapid method for the isolation and cultivation of tubercle bacilli directly from the sputum and feces, 38
- Phosphorus, chloroform, or alcohol, hepatic lesions of, and diet, 1
- Physiological function, histological structure, and localization of visual cortex, 617
- Pituitary body, influence of anterior lobe of, upon growth of carcinomata, 280
- Pneumococci in tuberculous sputum, types of, 146
- Pneumococcus, varieties of, and their relation to lobar pneumonia, 114
- Pneumonia, experimental, in rabbits, 304, 311, 320
- Pneumonia, experimental, in the rabbit, effect of sensitization on lesions of, 605
- Pneumonia, experimental, *intra vitam* staining in, and the circulation in the pneumonic lung, 311
- Pneumonia, lobar, production of, in rabbits, 304
- Pneumonia, lobar, relation of, to varieties of pneumococcus, 114
- Poisoning, benzol, factors of coagulation in experimental aplastic anemia of, with special reference to origin of prothrombin, 401
- Poisoning, late chloroform, resistance of pups to, in its relation to liver glycogen, 185
- Poisons, renal and hepatic, influence of diet upon necrosis caused by, 1, 21
- Polymyelitic tissues, survival and virulence of microorganism cultivated from, 91
- Polymyelitic virus, diffusion and survival of, 509
- Potassium chromate, uranium nitrate, or chloroform, nephritis caused by, and diet, 21
- Precipitation, specific, concentration of protective bodies in antipneumococcus serum by, 389
- Preservatives, functional effect of experimental intraspinal injections of sera with and without, 43
- PRINCE, ALEXANDER L. See BARBOUR and PRINCE, 330
- Proliferative power of normal and regenerating epithelial cells of the rat and guinea pig, 193
- Proteases, serum, and mechanism of Abderhalden reaction, 239
- Protective bodies in antipneumococcus serum, concentration of, by specific precipitation, 389
- Proteus*, *Bacillus*, pathogenic properties of, 629
- Prothrombin, factors of coagulation in experimental aplastic anemia of benzol poisoning, with special reference to origin of, 401
- Pure cultivation *in vivo* of vaccine virus free from bacteria, 539
- R**ED corpuscles, extracellular relation of the parasites to. Adult tertian malarial parasites attached to peripheral corpuscular mounds, 584
- Regenerating and normal epithelial cells of the rat and guinea pig, proliferative power of, 193
- Regeneration, epithelial, in reference to histogenesis of chronic uranium nephritis, 425
- Renal and hepatic poisons, influence of diet upon necrosis caused by, 1, 21
- RETTGER, LEO F. The influence of milk feeding on mortality and growth, and on the character of the intestinal flora, 365
- ROBERTSON, T. BRAILSFORD, and BURNETT, THEODORE C. The influence of the anterior lobe of the pituitary body upon the growth of carcinomata, 280
- Rous chicken sarcoma, effect of arsenic compounds on, 574
- S**ARCOMA, Rous chicken, effect of arsenic compounds on, 574
- Sensitization, effect of, on development of lesions of experimental pneumonia in the rabbit, 605
- Sera with and without preservatives, functional effect of experimental intraspinal injections of, 43
- Serum, antidyenteric, rapid production of, 515
- Serum, antipneumococcus, concentration of protective bodies in, by specific precipitation, 389
- Serum, antipneumococcus, distribution of immune bodies occurring in, 133
- Serum, influence of, upon trypanosomes, with special reference to its use for experiments *in vitro* with atoxyl and paraminophenylarsenoxide, 250
- Serum proteases and mechanism of Abderhalden reaction, 239
- SMYTH, HENRY FIELD. The reactions between bacteria and animal tissues

- under conditions of artificial cultivation, 103
- SPAIN, KATE C. The relation between the structure of the epidermis of the rat and the guinea pig, and the proliferative power of normal and regenerating epithelial cells of the same species, 193
- SPEED, J. S. See WHIPPLE and SPEED, 203
- Spleen, function of, in experimental infection of albino mice with *Bacillus tuberculosis*, 84
- Sputum and feces, new and rapid method for isolation and cultivation of tubercle bacilli directly from, 38
- Sputum, tuberculous, types of pneumococci in, 146
- Staining, *intra vitam*, in experimental pneumonia, and circulation in the pneumonic lung, 311
- Subcutaneous absorption and quantitative estimation of cholesterol, 179
- Survival and diffusion of poliomyelitic virus, 509
- Survival and virulence of microorganism cultivated from poliomyelitic tissues, 91
- T** wave of human electrocardiogram, influence of digitalis on, 593
- TERRY, B. T. The influence that serum exerts upon trypanosomes, with special reference to its use for experiments *in vitro* with atoxyl and paraminophenylarsenoxyl, 250
- Different amounts of transformed atoxyl produced by incubating 1 per cent. and 10 per cent. atoxyl in blood, 258
- The effect of heat on the transforming and binding power of blood, 267
- Tertian malarial parasites, adult, attached to peripheral corpuscular mounds. Extracellular relation of the parasites to the red corpuscles, 584
- Thyroid tissue in the guinea pig, comparison of autoplasmic and homeoplastic transplantation of, 164
- Tissue, fibrous, which appears in living cultures of adult frog tissues, origin and structure of, 455
- Tissue, thyroid, in the guinea pig, comparison of autoplasmic and homeoplastic transplantation of, 164
- Tissues, animal, and bacteria under conditions of artificial cultivation, reactions between, 103
- Tissues, human, various lesions of, wide-spread distribution of diphtheroids and their occurrence in, 493
- Tissues, poliomyelitic, survival and virulence of microorganism cultivated from, 91
- Transplantation, autoplasmic and homeoplastic, of thyroid tissue in the guinea pig, comparison of, 164
- Transplantation of tumors to foreign species, 571
- Treponema pallidum*, antibody formation against—agglutination, 576
- Treponema pallidum*, cultivation of, 213
- Trypanosoma lewisi*, changes in biological properties of, produced by experimental means, with special reference to virulence, 345
- Trypanosomes, influence of serum upon, with special reference to its use for experiments *in vitro* with atoxyl and paraminophenylarsenoxyl, 250
- Tubercle bacilli, new and rapid method for isolation and cultivation of, directly from sputum and feces, 38
- Tuberculosis*, *Bacillus*, experimental infection of albino mice with, function of spleen in, 84
- Tuberculous sputum, types of pneumococci in, 146
- Tumor growth, mode of action of substances inhibiting, and immunization against these substances, 155
- Tumors, transplantation of, to foreign species, 571
- U** RANIUM nephritis, chronic, histogenesis of, with especial reference to epithelial regeneration, 425
- Uranium nitrate, potassium chromate, or chloroform, nephritis caused by, and diet, 21
- V** ACCINE virus free from bacteria, pure cultivation *in vivo* of, 539
- Virulence and changes in biological properties of *Trypanosoma lewisi* produced by experimental means, 345
- Virulence and survival of microorganism cultivated from poliomyelitic tissues, 91
- Virus, poliomyelitic, diffusion and survival of, 509
- Virus, vaccine, free from bacteria, pure cultivation *in vivo* of, 539
- Visual cortex, its histological struc-

- ture, localization, and physiological function, 617
- WADE, H. WINDSOR. See HARRIS and WADE, 493
- WHIPPLE, G. H., and SPEED, J. S. Liver function as influenced by anesthetics and narcotics, 203
- WINTERNITZ, M. C., and KLINE, B. S. Studies upon experimental pneumonia in rabbits. IX. The part of the leucocyte in the immunity reaction, 320
- WINTERNITZ, M. C. See KLINE and WINTERNITZ, 304, 311
- ZINSSER, HANS, and HOPKINS, JOSEPH GARDNER. Antibody formation against *Treponema pallidum*—agglutination, 576
- ZINSSER, HANS, HOPKINS, J. G., and GILBERT, RUTH. Notes on the cultivation of *Treponema pallidum*, 213





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